Theobromine

Theobromine:

effects on postprandial metabolism, vascular function and intestinal gene expression in humans

Lotte Smolders

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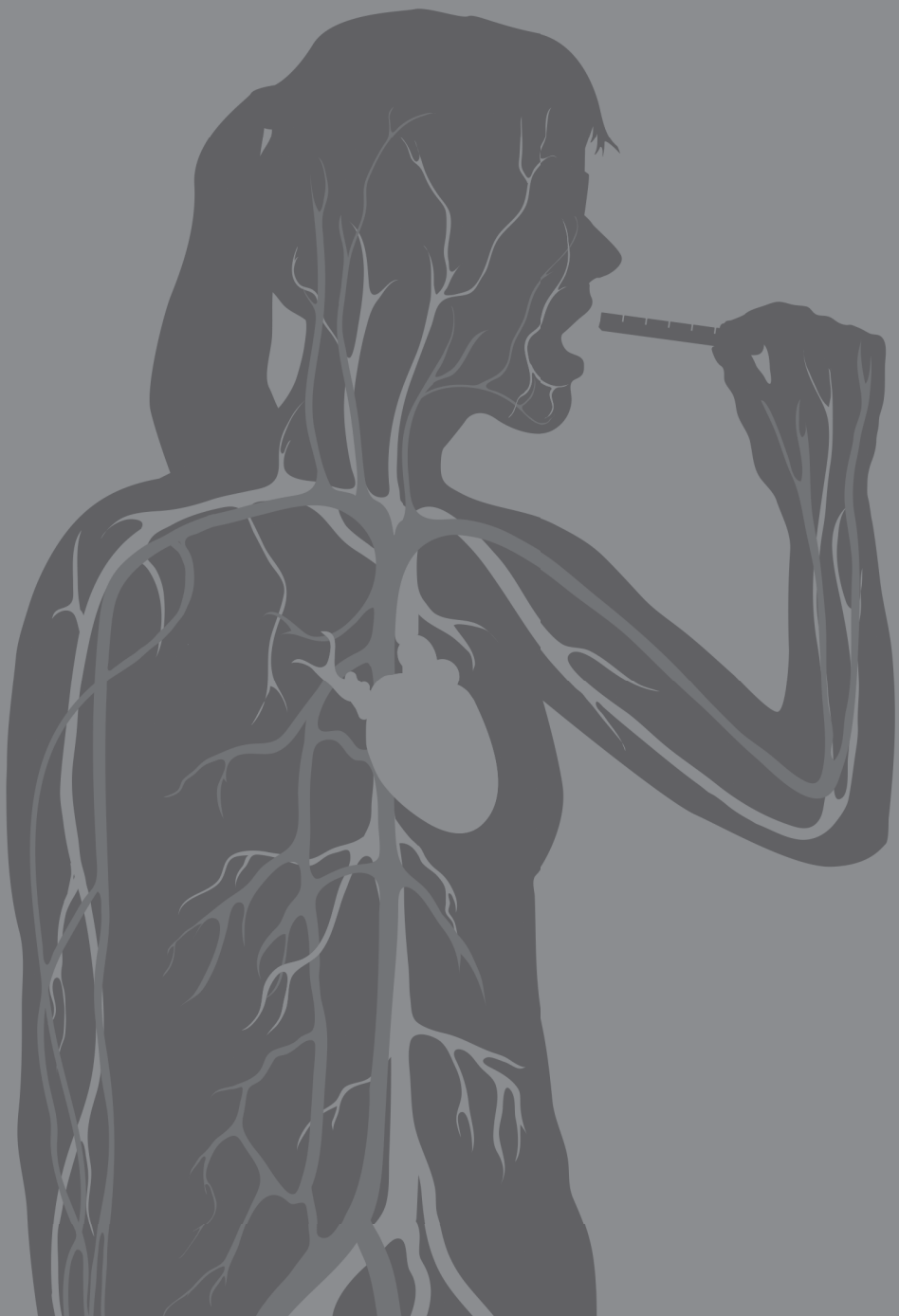
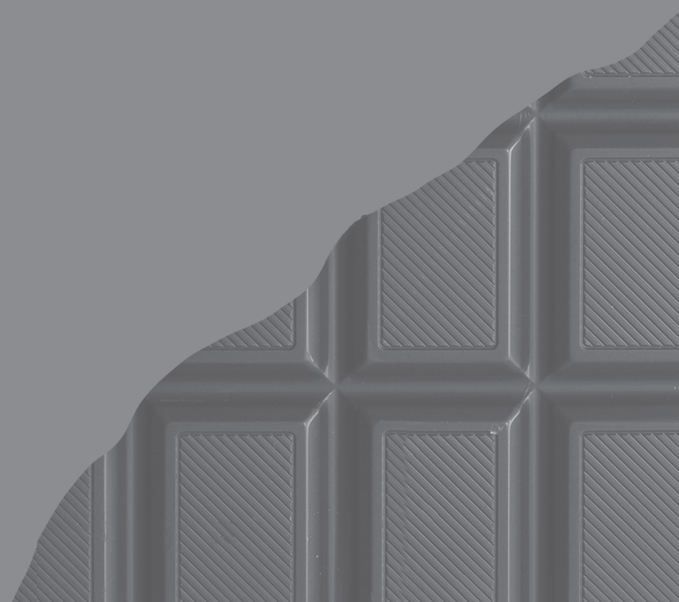


Chapter 1

General introduction



Cardiovascular diseases

Despite excellent pharmacological treatment possibilities, cardiovascular diseases (CVD) are still the leading cause of death worldwide.[1] CVD are caused by disorders of the blood vessels and the heart and involve a group of syndromes, including cerebrovascular disease, coronary heart disease (CHD), and peripheral arterial disease. Many pharmacological treatment options are focused on lowering the risk for CHD by decreasing serum low density lipoprotein (LDL) cholesterol (LDL-C) concentrations. However, despite successful intervention strategies, there is still a substantial residual cardiovascular risk.[2] This clearly indicates the need for other approaches to prevent the initiation and progression of this disease. Therefore, other pathways to lower CHD risk needs to be investigated. Increasing serum high density lipoprotein (HDL) cholesterol (HDL-C) concentrations was thought to be promising, since these are inversely associated with CVD risk.[3] However, several clinical studies failed to show cardioprotective effects of actively increasing serum HDL-C concentrations.[4-6] New evidence, however, suggests that the focus should be on optimizing HDL functionality instead of increasing serum HDL-C concentrations itself.[7]

HDL functionality

Optimized HDL functionality is related to HDL particles that have the ability to take up more cholesterol from peripheral tissues, leading to an increased cholesterol efflux.[8] The major protein in HDL particles is apolipoprotein A-I (apoA-I).[9] Epidemiological studies have shown that not only higher serum HDL-C but also higher serum apoA-I concentrations are associated with a lower CVD risk.[10] Furthermore, apoA-I is correlated with cholesterol efflux capacity.[11] It is now postulated that raising apoA-I production might improve HDL functionality and therefore protect against CVD development.[8] In this respect, diet may play an important role, as recent studies have indicated a relation between diet and cholesterol efflux capacity.[12,13]

ApoA-I synthesis and clearance is briefly discussed in **chapter 2**. In addition, possibilities to specifically optimize apoA-I metabolism by using dietary or novel pharmacological interventions is systematically reviewed. Only few dietary compounds were found to increase fasting apoA-I concentrations. One of these components was theobromine. Theobromine is found in cocoa and may have several health benefits.[14] In this dissertation we will therefore focus on the effects of adding theobromine to our habitual diet.

Theobromine

Several studies have shown beneficial effects of cocoa or dark chocolate consumption on circulating lipids, lipoproteins[14,15] and other CVD risk markers.[16] It is possible that these positive effects can be explained by theobromine, which is also a metabolite of caffeine. Dietary intake of theobromine is low; 50-100 g cocoa contains only 800-1500 mg theobromine,[17] whereas 50 g dark chocolate contains 240-520 mg theobromine.[18] Theobromine is effectively absorbed in the intestine[19,20] and reaches the portal vein by diffusion through the enterocytes.[19] In the portal vein, theobromine is bound to the carrier protein Human Serum Albumin, via which it is transported to the liver.[21] Maximal serum concentrations of theobromine are reached 2 hours post-ingestion[19] and the half-life in the circulation is 6–10 hours.[19,22] In the liver, theobromine is metabolized by 2 cytochrome P450 enzymes: CYP1A2 and CYP2E1.[23] The main metabolites of theobromine are 7-methylxanthine, 3-methylxanthine, and 3,7-dimethyluric acid (Figure 1).[23] Theobromine and its metabolites are extracted by the kidneys and excreted via the urine.[19,20,24]

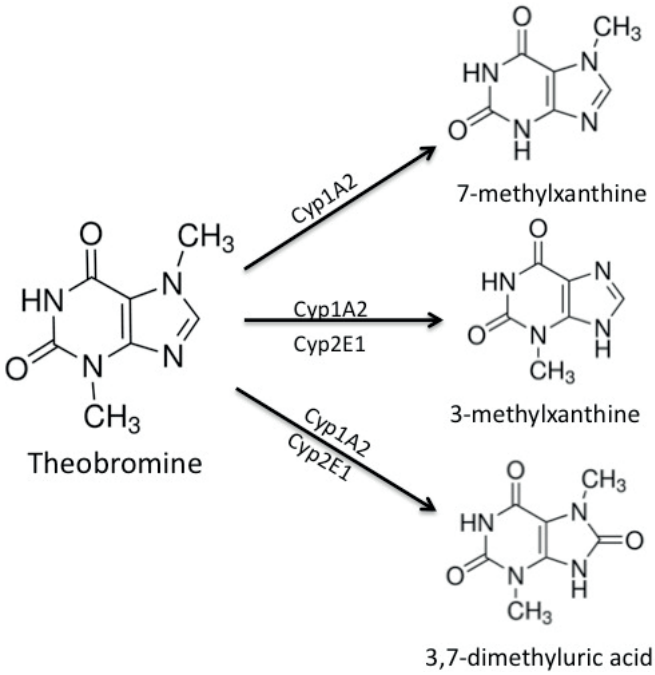


Figure 1 *Metabolism of theobromine by cytochrome P450 enzymes*

So far, the effects of pure theobromine have only been evaluated in one study. In healthy volunteers, 850 mg of theobromine for 4-weeks significantly increased serum HDL-C and apoA-I concentrations and decreased serum LDL-C and apolipoprotein B100 (apoB100) concentrations.[25] Because of these promising effects of theobromine on apoA-I and circulating fasting lipids, effects of theobromine on postprandial metabolism and vascular function were examined in this dissertation (**Chapter 3, 5 and 6**). Furthermore, underlying mechanisms of the effects of theobromine were investigated by studying changes in intestinal gene expression (**Chapter 4 and 5**).

Postprandial metabolism

Increasing evidence suggests that not only fasting lipid, lipoprotein and glucose concentrations, but also a disturbed postprandial triacylglycerol (TAG) and glucose metabolism are important risk markers for CVD.[26] Furthermore, in the Western world the majority of the population spends a significant part of the day in the postprandial state. Therefore, we evaluated in two clinical studies the effects of acute (**Chapter 3**) and 4-weeks of theobromine consumption on postprandial lipid, lipoprotein and glucose metabolism (**Chapter 5**).

Intestinal gene expression

Theobromine is absorbed in the intestine[19,20] and metabolized by the liver.[23] Also apoA-I is produced in the cells of the small intestine and the liver.[27] To unravel underlying mechanisms of theobromine on apoA-I concentrations, we also investigated whether potential effects of theobromine on apoA-I concentrations were related to a higher de novo apoA-I production. For this, changes in gene expression in human duodenal biopsies were investigated, using microarray analysis after acute (**Chapter 4**) and 4-weeks of theobromine consumption (**Chapter 5**).

Vascular function

Whether optimizing HDL functionality indeed translates into improved CHD outcomes such as myocardial infarction, atherosclerosis, and total mortality, has hardly been studied. However, novel surrogate risk markers related to CVD risk exist, such as vascular function markers,[28-32] which are easier to study and have been related to CVD risk. Several of these markers reflecting vascular function, each addressing different aspect of the vasculature, were measured as read out parameters in one of the theobromine intervention studies as described in this thesis (**Chapter 6**).

Endothelial function

The endothelium is the inner lining of the blood vessels and has several important functions, including the regulation of the vascular tone, hemostasis, angiogenesis and

inflammatory processes.[33] A dysfunction of this endothelium is the first steps towards atherosclerosis. However, in the early stage, dysfunctioning of the endothelium can still be reversed.[34]

Brachial artery flow mediated dilatation (FMD) is the current gold standard to non-invasively measure endothelial function.[35] During this measurement, reactive hyperemia is induced by the inflation of a forearm cuff. By release of this cuff, shear stress occurs, after which a blood vessel dilates, mediated by the release of nitric oxide (NO) from the endothelium (Figure 2). A blood vessel with dysfunctional endothelium will show a blunted response in comparison with a blood vessel with a healthy, more functional endothelium. Endothelial dysfunction, as measured with FMD, is a predictor for future CVD events.[30] Although FMD is currently considered as the gold standard, it is operator specific and need extensive training for proper and reliable measurements. An easier way to measure endothelial function is via peripheral arterial tonometry (PAT). This measurement records changes in arterial pulse wave amplitude in the fingertip, following reactive hyperaemia caused by the release of a forearm cuff. The change in arterial pulse wave amplitude after this reactive hyperaemia is called the reactive hyperaemia index (RHI) and is a measure for endothelial function (Figure 2). In comparison to the FMD, this measurement is more related to the endothelial function of small arteries and of the microcirculation. A reduced RHI is related to the presence of CVD risk factors.[29]

Arterial stiffness

Arterial stiffness results from a degenerative process affecting mainly the extracellular matrix of elastic arteries. Changes in extracellular matrix proteins and in the mechanical properties of the vessel wall related to arterial stiffening may lead to atherosclerosis.[36]

Pulse wave velocity of the carotid to femoral (PWVcf) artery is the gold standard to measure arterial stiffness.[37] It measures the speed of a pulse pressure wave, traveling trough the arterial tree (Figure 2). An increased PWVcf, is a predictor for a higher frequency of stroke, CVD and total mortality.[31] Another measure reflecting arterial stiffness is the augmentation index (AIx). This index is defined as the difference between the first and the second peak of the arterial waveform, the wave reflection (Figure 2). A higher AIx indicates stiffer vessels, and is associated with higher CVD risk.[32]

Microvasculature

Characteristics of the microvasculature in relation to CVD risk can be studied by measuring the arteriolar and venular width of the blood vessels in the retina (Figure 2). Cross-sectionally wider venules and narrower arterioles are associated with

an increased risk of CVD events in women, but not in men.[28] Furthermore, larger venular diameters are independently associated with lower HDL-C concentrations[38] and more inflammation,[39] while a higher blood pressure is a systemic determinant for smaller arteriolar calibers.[40]

Postprandial vascular function

It is known that both postprandial hyperlipidemia[41] and hyperglycemia[42] impair vascular function. Therefore, it is important not only to focus on nutritional strategies to improve fasting vascular function, but also to identify strategies that counterbalance impaired postprandial vascular function. A high fat mixed meal can be used as a physiological stressor, because it impairs postprandial endothelial function.[43-45] Furthermore, high fat meals apparently improve AIx,[44,46] while results on PWV are conflicting[46-48] and effects on arteriolar and venular calibers have hardly been studied (Figure 3). We therefore evaluated in a randomized human placebo controlled trial the effects of 4-weeks of theobromine consumption on vascular function in fasting state and after a high fat mixed meal challenge (**Chapter 6**).

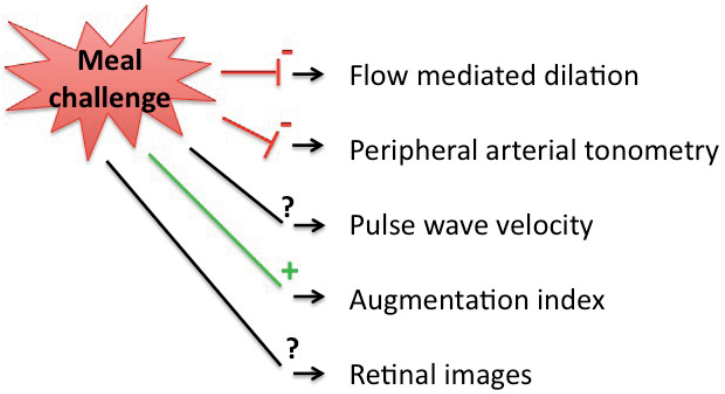


Figure 3 *Effects of a high fat meal challenge on parameters reflecting vascular function*

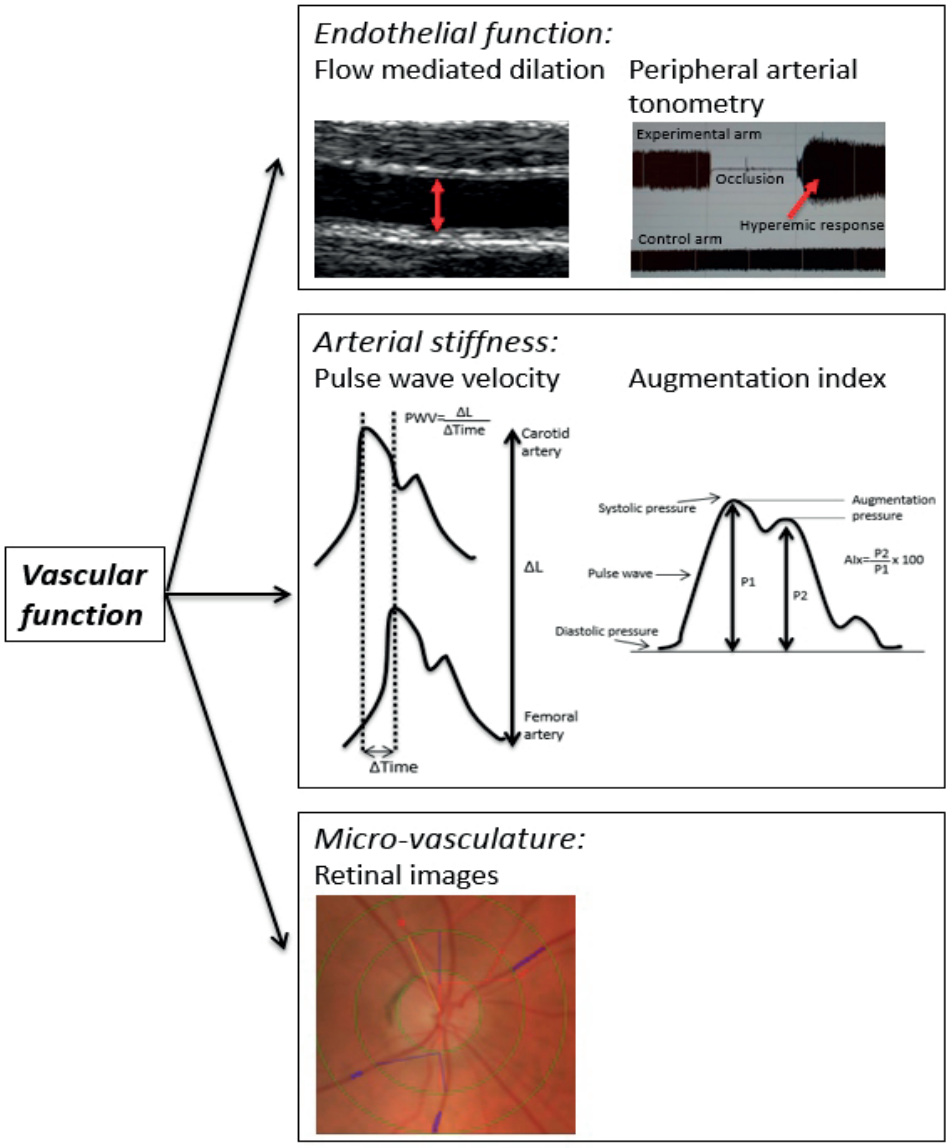


Figure 2 *Vascular function measurements*

Theobromine vs. fat on apoA-I metabolism

From the systematic review presented in **chapter 2**, it was concluded that only a few foods or dietary components might increase serum apoA-I concentrations. One well-studied macronutrient that can increase fasting serum apoA-I concentrations is fat.[49] Two recent meta-analyses showed that the exchange of carbohydrates for saturated, unsaturated or trans fatty acids increased fasting apoA-I concentrations.[49,50] Therefore, in our first intervention study a meal high in fat was used as comparison for the effects of theobromine on apoA-I metabolism. Till now, only few studies have investigated the acute effects of a high fat meal on apoA-I concentrations. Furthermore, underlying mechanisms of the increase in apoA-I concentrations due to a high fat meal have not been studied before into great detail. Therefore, the effects of an acute high fat / low carbohydrate and an acute low fat / high carbohydrate meal on postprandial metabolism (**Chapter 3**) and duodenal gene expression were investigated (**Chapter 4**).

Thesis outline

The aim of this thesis was to investigate the effects of theobromine on postprandial metabolism, vascular function and intestinal gene expression in humans. In addition, it is of interest to investigate if theobromine is the component from cocoa that relates to the positive effects on lipid metabolism and on CVD risk. For this, a systematic review was written and two human intervention studies were performed. First, **chapter 2** summarizes the effects of dietary or novel pharmacological interventions on apoA-I metabolism. It was concluded, that only few foods and dietary components might increase apoA-I concentrations, including theobromine. Next, **chapter 3 and 4** describe the results of the first human intervention study in which the acute effects of theobromine on postprandial lipid, lipoprotein and glucose metabolism (**Chapter 3**) as well as on duodenal gene expression (**Chapter 4**) were investigated. Then, in **chapter 5 and 6** the results of the second human intervention study investigating the effects of 4-weeks theobromine consumption on cardiometabolic risk markers are described. **Chapter 5** focuses on the effects of theobromine on fasting and postprandial metabolism combined with the analysis of duodenal gene expression, while **chapter 6** describes the effects of theobromine on fasting and postprandial vascular function. Finally, **chapter 7** summarizes the major findings of the different studies in the present thesis and the results are discussed in the context of the health benefits of cocoa.

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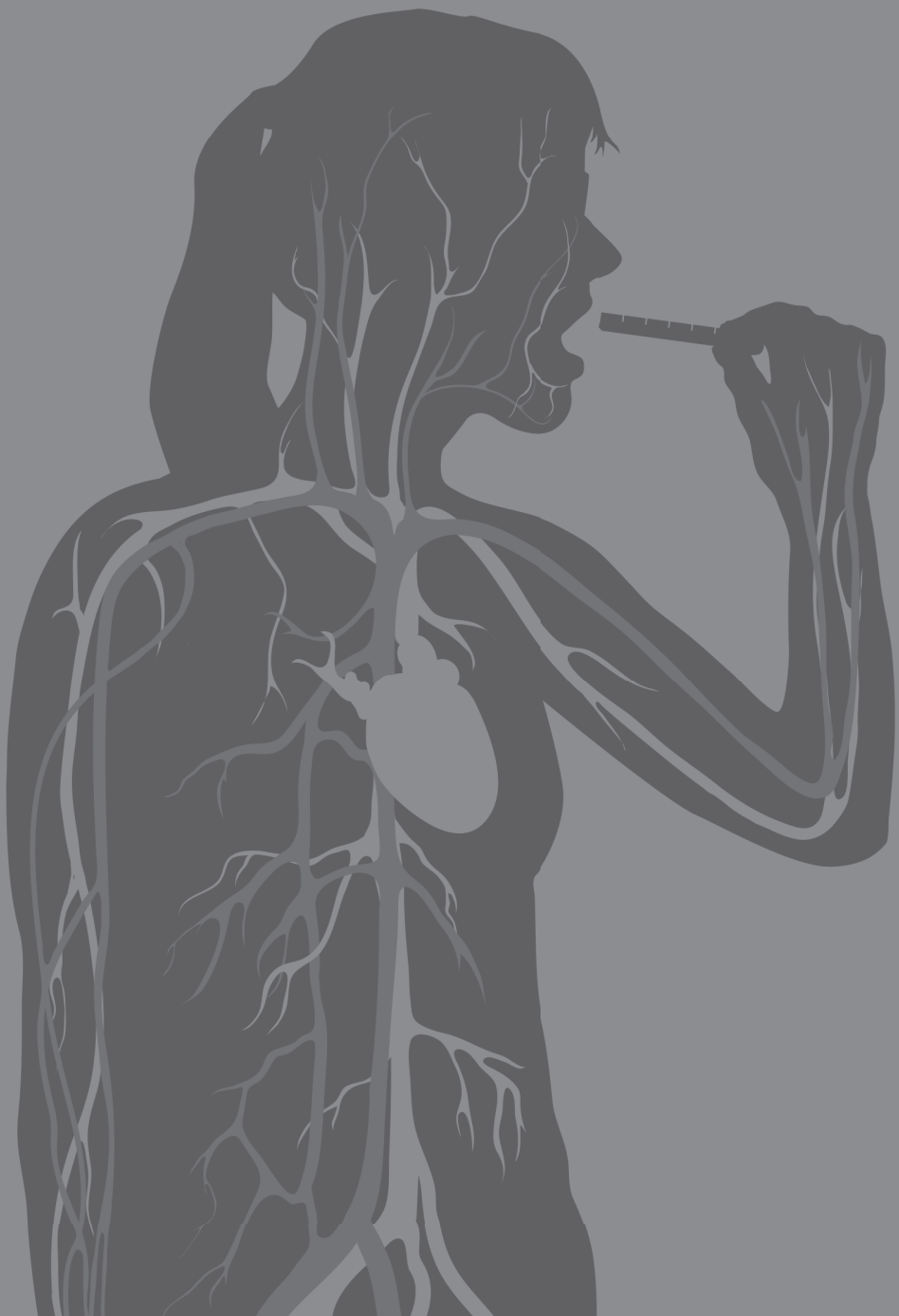

Chapter 2

Dietary strategies and novel pharmaceutical approaches targeting serum apoA-I metabolism: a systematic overview

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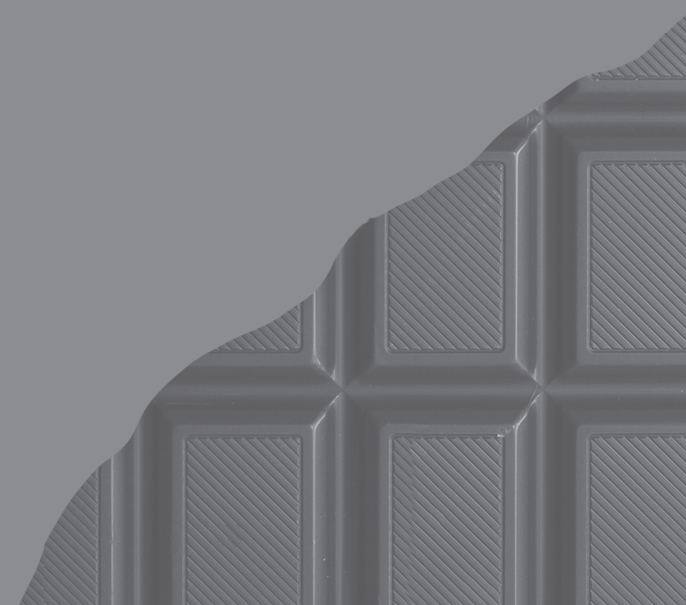

Abstract

The incidence of coronary heart diseases (CHD) is still increasing, which underscores the need for new preventive and therapeutic approaches to decrease CHD risk. In this respect, increasing apoA-I concentrations may be a promising approach, especially through increasing apoA-I synthesis. This review first provides insight into current knowledge on apoA-I production, clearance and degradation, followed by a systematic review of dietary and novel pharmacological approaches to target apoA-I metabolism. For this, a systematic search was performed to identify randomized controlled intervention studies that examined effects of whole foods and (non) nutrients on apoA-I metabolism. In addition, novel pharmacological approaches were searched for, that were specifically developed to target apoA-I metabolism. We conclude that both dietary components and pharmacological approaches can be used to increase apoA-I concentrations or functionality. For the dietary components in particular, more knowledge about the underlying mechanisms is necessary, as increasing apoA-I per se does not necessarily translate into a reduced CHD risk.

Background

The global incidence of coronary heart diseases (CHD) is still increasing, which underscores the need for novel and alternative approaches to prevent the initiation and progression of this disease already at an early stage. Since elevated serum low-density lipoprotein cholesterol (LDL-C) concentrations are causally related to CHD, most dietary life style interventions and pharmaceutical treatments to prevent CHD so far are focused on lowering serum LDL-C concentrations. Despite successful intervention possibilities, there is still a substantial residual cardiovascular risk. Therefore, a possibility to further lower CHD risk is to target multiple metabolic pathways simultaneously.[1,2] For example, statin treatment, to lower serum LDL-C concentrations, can be combined with other pharmaceutical agents, such as proprotein convertase subtilisin/kexin type 9 inhibitors, which substantially further lower serum LDL-C concentrations.[3] Also, the Niemann-Pick Like Intracellular Cholesterol Transporter 1 inhibitor ezetimibe can be used, which has been shown to further lower the number of myocardial infarctions with 13%, strokes with 14% and ischemic strokes with 21%.[4] Besides combined interventions to further increase the LDL-C lowering potential, it can be considered to mutually target at the same time other CHD risk parameters including serum high density lipoprotein (HDL) cholesterol (HDL-C), apolipoprotein A-I (apoA-I), triacylglycerol or lipoprotein(a) concentrations and/or blood pressure.[5] These parameters may be interrelated. An inverse relationship exists, for example, between serum triacylglycerol and HDL-C concentrations. Thus, interventions that change triacylglycerol may therefore also affect HDL metabolism. In this review we will however focus on possibilities to further reduce CHD risk via novel and alternative dietary and pharmacological interventions targeting apoA-I metabolism.

Increasing HDL functionality by increasing apoA-I

So far, interventions specifically targeting to increase serum HDL-C concentrations did not report any protective cardiovascular effect, which has clearly negatively influenced the interest to develop novel interventions to elevate serum HDL-C. However, recent evidence suggests that the focus should be on optimizing HDL functionality instead of elevating circulating serum HDL-C concentrations.[6] By increasing their functionality, HDL particles are able to take up more cholesterol from peripheral tissues; i.e. the so-called cholesterol efflux. In addition, a more functional HDL particle will be more anti-oxidative - in particular by inhibiting LDL oxidation - and more anti-thrombotic, and will have a higher anti-inflammatory and anti-apoptotic activity.[7] A wealth of evidence from epidemiological, in vitro and in vivo studies suggests that higher apoA-I concentrations protect against CHD

development.[8] By increasing apoA-I concentrations, the resulting newly produced small HDL particles (i.e. pre-beta HDL) will be highly functional, thereby enhancing cholesterol efflux.[7] Indeed, it has been found that apoA-I concentration is the strongest predictor for cholesterol efflux capacity.[9] ApoA-I is the major protein of HDL particles[10] contributing to approximately 33% of the total HDL particle mass, and up to 60% of the HDL protein mass.[11] The most likely mechanism explaining the beneficial effects of elevated serum apoA-I concentrations origins from the fact that apoA-I is the ligand for ATP-binding cassette transporter A1 (ABCA1), as such mediating cholesterol efflux from lipid-loaden macrophages.[7] Based on this information, Smits et al. wrote a clear plead for strategies to increase serum apoA-I concentrations as the most promising target for enhancing HDL functionality, thereby decreasing cardiovascular disease (CVD) risk.[12] However, lowering CHD risk by increasing endogenous apoA-I production, by decreasing apoA-I degradation, or by providing exogenous apoA-I has for unknown reasons not yet been investigated into great detail. Therefore, the question remains whether specifically targeting apoA-I metabolism is a suitable target to reduce CHD risk.

In this review we will first briefly provide insight into the current knowledge of apoA-I synthesis, clearance and degradation, followed by a detailed overview of dietary and novel experimental pharmaceutical developments targeting circulating apoA-I concentrations.

ApoA-I

ApoA-I synthesis

ApoA-I mRNA is expressed in cells of the liver and small intestine,[13] where it is translated into a pre-pro-apoA-I protein. The pre-segment needs co-translational cleavage,[14] which takes place during translocation of the protein into the endoplasmatic reticulum by a signal peptidase.[15,16] This results in a stable intracellular pro-apoA-I protein,[14] which is secreted into blood and lymph. Directly after secretion of pro-apoA-I, the pro-protein is cleaved of by Bone Morphogenetic Protein-1 (BMP-1) and Procollagen C-proteinase Enhancer-2 Protein (PCPE2) (Figure 1).[17,18] It is evident that the cleavage of the pro-segment is essential for the secretion of newly formed intracellular apoA-I. Deletion of the coding sequence of the pro-segment causes accumulation of apoA-I in the cell,[19] decreases the efficiency of apoA-I mRNA expression,[16] and impairs the secretion of apoA-I into blood and lymph.[16,19] The cleavage of the pro-protein occurs relatively rapid, while the residence time for pro-apoA-I in plasma is only 5.5 hours, in contrast to the residence time for mature apoA-I of 6.5 days.[20] About 4-8% of the circulating apoA-I pool is pro-apoA-I.[14,21,22] After cleavage of the pro segment, apoA-I accepts cholesterol and phospholipids from ABCA1[23] to form a pre-β HDL particle

(Figure 1). In other words, apoA-I is the starting point for the synthesis of a functional HDL particle and therefore essential for the formation and maturation of novel HDL particles.[15] In the circulation, lecithin:cholesterol acyltransferase esterifies the free cholesterol in these pre-β HDL particles, thereby forming HDL_3 and finally HDL_2.[24] The ATP binding cassette G1 transporter and scavenger receptor class B type 1 (SR-B1) contribute to the cholesterol efflux from peripheral tissues and macrophages to these mature HDL particles. After binding of HDL_2 to SR-B1 on the liver, cholesterol esters are taken up and lipid-depleted apoA-I is returned to the circulation. These apoA-I-rich lipid-depleted HDL particles can again acquire cholesterol and phospholipids forming an pre-β HDL particle or can be cleared from the circulation.[25]

ApoA-I clearance

Several organs are involved in apoA-I clearance and degradation.[25] Calculations in rabbits have indicated that renal apoA-I clearance accounts for approximately 68-70% of total apoA-I catabolism. Also in humans, the kidney is the major site for apoA-I clearance.[25,26] In the kidneys, the uptake of HDL particles is limited, because the intact lipoprotein particles are too large to pass the glomerular filtration barrier. However, newly formed or recycled lipid free apoA-I, can pass this barrier. In the proximal tubule of the glomerulus apoA-I binds the receptors cubilin and megalin,[27] which mediate endocytosis and delivery of the protein to the lysosomes,[28,29] resulting in complete degradation of the apoA-I protein. The amino acids can be re-used for de novo protein synthesis.[30] While the kidneys plays a major role in apoA-I degradation, the liver accounts for 26% of the apoA-I clearance, at least in rats. It is not known how the hepatocytes take up the apoA-I particles. The apoA-I catabolic products are excreted from the liver via the bile into the gut, where they are further digested and absorbed, or excreted from the body. Other tissues, besides kidney and liver, which are to a lesser extent involved in the degradation of apoA-I are ovaries, adrenals and spleen, which secrete apoA-I catabolic products into the urine (Figure 1).[25]

Increasing apoA-I concentrations via reducing apoA-I clearance is for unknown reasons not a subject of investigation. Consequently, it is also not known whether inhibiting apoA-I clearance, affects HDL functionality. Therefore, decreasing apoA-I clearance currently not a target for interventions, whereas elevating de novo apoA-I production certainly is.[31]

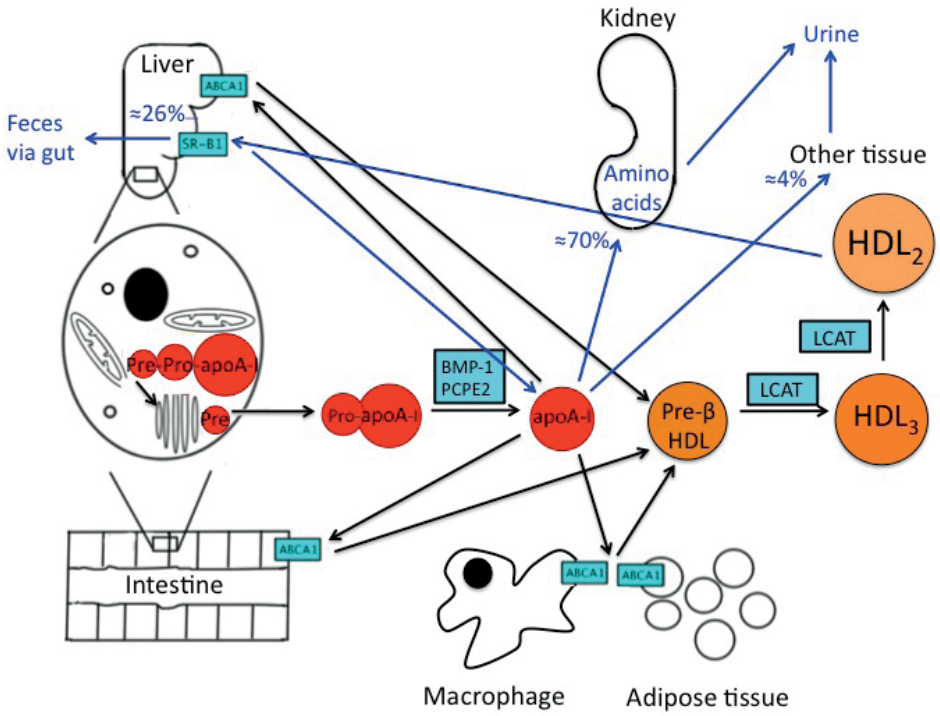

Figure 1 *Simplified scheme of the synthesis, metabolism and clearance of apoA-I. ApoA-I is synthesized in cells of the liver and intestine as pre-pro-apoA-I. After translocation to the endoplasmic reticulum, the pre-protein is cleaved of and pro-apoA-I is secreted into blood and lymph. In the circulation, the pro segment is directly cleaved of by Bone Morphogenetic Protein-1 (BMP-1) and Procollagen C-proteinase Enhancer-2 Protein (PCPE2). After this, apoA-I accepts cholesterol and phospholipids from ABCA1, forming a pre-β HDL particle. In the circulation, lecithin:cholesterol acyltransferase (LCAT) esterifies the free cholesterol in these pre-β HDL particles, forming HDL_3 and finally HDL_2, as indicated by the black arrows. After binding of HDL_2 to SR-B1 on the liver, the cholesterol esters are taken up and lipid-depleted apoA-I is returned to the circulation. These apoA-I-rich particles can again acquire cholesterol and phospholipids or can be cleared from the circulation. Clearance will take place for 70% by the kidney, where apoA-I is broken down into amino acids and ultimately excreted in the urine. 26% of the free apoA-I will be cleared by the liver, and apoA-I catabolic products will then be excreted via biliary secretion into the gut, and further digested and absorbed, or excreted from the body through the feces. 4% of the free apoA-I will go to other tissues and finally will end up in the urine, as indicated by the blue arrows.*

Dietary interventions affecting apoA-I metabolism

It has been clearly shown that dietary components can change serum apoA-I concentrations. We here provide an overview of randomized controlled dietary intervention studies that have examined the effects of whole foods and (non)nutrients on apoA-I concentrations or apoA-I metabolism. Only crossover and parallel studies were included. Potentially relevant studies published before January 2017 were

identified by a systematic search of the database PubMed (www.ncbi.nlm.nih.gov). The following search terms were used to search in titles and abstracts: (((Clinical Trial[Publication Type]) OR randomized controlled trial[Publication Type])) AND apolipoprotein A*[MeSH Terms]. The selection was performed in two steps. First, titles and abstracts were screened. Studies were selected if they met the following inclusion criteria: human intervention study with adults, dietary intervention study, and measurement of apoA-I concentrations. In the second step, full-texts of the selected articles were read to extract fasting or postprandial apoA-I values. Then, a search was performed to find meta-analysis of each food or (non)nutrient group. When a meta-analysis was found, it is included in this review together with the articles identified by us, but which were not part of the meta-analysis. Changes in apoA-I concentrations were expressed as percentages, if possible. When percentages were not reported, they were calculated from the mean values as reported in the articles. Furthermore, the list of articles was screened for studies that investigated the effects on cholesterol efflux, apoA-I production rate (PR), or fractional catabolic rate (FCR).

Alcohol

Based on a meta-analysis including 16 studies with in total 374 subjects, Brien et al. concluded that alcohol consumption (women: >15 g alcohol/day, men: >30 g alcohol/day) increased fasting plasma apoA-I concentrations with 10.1 mg/dL (95% CI 7.3 - 12.9 mg/dL).[32] A later study, not included in this meta-analysis, also showed a higher fasting apoA-I concentration after alcohol consumption as compared with no alcohol consumption.[33] Moreover, postprandial apoA-I concentrations also increased after alcohol consumption.[34] These effects did not depend on the source (red wine, beer, Dutch gin) of alcohol.[35] Lavy et al. however, reported that red wine increased apoA-I as compared with white wine consumption.[36] Also, Gepner et al. observed that red wine increased apoA-I concentrations as compared with water consumption, but white wine did not significantly change apoA-I concentrations as compared with water or red wine.[37] Furthermore, alcohol consumption not only elevated circulating apoA-I concentrations but also improved HDL-functionality as shown by an increased cholesterol efflux capacity.[35,38,39] In one study, the kinetics of apoA-I have been examined. It was reported that apoA-I PR increased and apoA-I FCR decreased after alcohol consumption (Table 1).[40]

Boiled and filtered coffee, caffeine and tea

In six studies, the effects of boiled or filtered coffee, cafeïne and tea on fasting apoA-I concentrations have been compared. In none of the studies, significant differences in apoA-I concentrations were observed (Table 2).[41-46]

Table 1 *Effect of alcohol consumption on apoA-I concentrations, HDL-functionality and apoA-I kinetics*

First author, year	Food component/ product	Study design and duration	Participants	Intake	Effects
Brien et al. (2011)[32]	Alcohol	Meta-analysis of 16 studies till 2009: - RCT with 2 arms - before vs. after > 1 week	374 subjects	Women >15 g/day Men >30 g/day	- 10.1 mg/dL (95% CI 7.3 - 12.9) ↓ in fasting plasma apoA-I concentrations
Lavy et al. (1994)[36]	Red vs. white wine	RCT parallel 2 weeks	20 healthy men	44 g alcohol/day	- 12.0% ↓ in fasting plasma apoA-I concentrations comparing red wine with white wine
Van der Gaag et al. (1999)[34]	Red wine vs. beer vs. Dutch gin vs. water	RCT cross over 3 weeks	11 healthy men	40 g alcohol/day from red wine, beer or Dutch gin	- 8.2% ↓ in fasting serum apoA-I concentrations comparing alcohol with water * - 9.2% ↓ in postprandial serum apoA-I concentrations comparing alcohol with water * - No differences between the different beverages
Van der Gaag et al. (2001)[35]	Red wine vs. beer vs. Dutch gin vs. water	RCT cross over 3 weeks	11 healthy men	40 g alcohol/day from red wine, beer or Dutch gin	- 10% ↓ in fasting plasma apoA-I concentrations comparing alcohol with water * - 6.2% ↓ in cholesterol efflux comparing alcohol with water * - No differences between the different beverages
Beulens et al. (2004)[38]	Whisky vs. water	RCT cross over 17 days	23 healthy men	40 g alcohol/day	- 6.2% ↓ in fasting plasma apoA-I concentrations - 17.5% ↓ in cholesterol efflux
Kralova Lesna et al. (2009)[39]	Beer vs. non alcoholic beverage	RCT cross over 4 weeks	13 healthy men	36 g alcohol/day	- 7.5% ↓ in fasting plasma apoA-I concentrations * - 8.0% ↓ in fasting cholesterol efflux *
Gepner et al. (2015)[37]	Red vs. white wine vs. water	RCT parallel 2 years	195 patients with diabetes mellitus type 2	17 g alcohol/day	- 2.3% ↓ in fasting plasma apoA-I concentrations comparing red wine with water * - No difference between white wine and water or red wine
Chiva-Blanch et al. (2013)[33]	Red wine vs. dealcoholized red wine vs. gin	RCT cross over 4 weeks	67 men at high CVD risk	30 g alcohol/day	- 12.5% and 12.6% ↓ in fasting plasma apoA-I concentrations comparing dealcoholized red wine with red wine and gin respectively *
Gottrand et al. (1999)[40]	Red wine vs. non alcoholic beverage	RCT cross over 4 weeks	5 healthy men	50 g alcohol/day	- 20% ↓ in plasma apoA-I pool - 10% ↓ in PR - 6% ↑ in FCR

** Percentages calculated from the mean values, PR: production rate, FCR: fractional catabolic rate*

Fatty acids

In a recent meta-analysis including 104 diets from forty-two well-controlled intervention studies the effects of the various fatty acids on fasting serum apoA-I concentrations were estimated. Effects of fish fatty acids were not included in that meta-analysis and will be discussed in the next paragraph. A significant increase in serum apoA-I concentrations was found when 1 energy % of carbohydrates was replaced by saturated fatty acids (SFA; 8.4 mg/dL, 95% CI 6.4 - 10.5), cis-monounsaturated fatty acids (cis-MUFA; 5.5 mg/dL, 95% CI 3.7 - 7.3) and cis-polyunsaturated fatty acids (cis-PUFA; 2.3 mg/dL, 95% CI 0.1 - 4.6). Cis-MUFA mainly referred to oleic acid and cis-PUFA to linoleic acid plus some α-linolenic acid. This meta-analysis further showed that fasting apoA-I concentrations were significantly increased by replacement of 1 energy % from carbohydrates with lauric acid (C12:0; 19.2 mg/dL, 95% CI 14.6 - 12.7), myristic acid (C14:0; 8.8 mg/dL, 95% CI 0.5 - 13.1) and palmitic acid (C16:0; 6.5 mg/dL, 95% CI 3.8 - 9.3), while replacement with stearic acid (C18:0) did not change apoA-I concentrations. For these latter analyses, 88 diets from 34 studies were included.[47] In another meta-analysis based on 17 diets from 10 studies, Brouwer (2016) described the effects of trans fatty acids (TFA) on circulating fasting apoA-I concentrations. It was reported that replacement of 1 energy % of carbohydrates for total TFA increased apoA-I concentrations (3.3 mg/dL, 95% CI 4.7 - 1.9). When a difference was made between industrial and ruminant TFA, it was found that replacement with industrial TFA significantly increased fasting apoA-I concentrations (3.3 mg/dL, 95% CI 4.8 - 1.8), while ruminant TFA did not (4.6 mg/dL, 95% CI: –22.0 - 12.9). This may be due to a lack of power, since only two studies investigated ruminant TFA. Furthermore, this meta-analysis also showed that replacement of 1 energy % from TFA with SFA increased fasting apoA-I concentrations (2.6 mg/dL, 95% CI 1.4 - 3.9), while replacement with MUFA did not change apoA-I concentrations and replacement with cis-PUFA decreased fasting apoA-I concentrations (-1.7 mg/dL, 95% CI -2.8 - -0.6) (Table 3).[48]

Several studies have examined the effects of the various fatty acids on serum apoA-I metabolism. A TFA diet increased apoA-I FCR as compared with SFA, but the FCR after cis-PUFA consumption did not differ from the TFA or SFA diets. ApoA-I PR was not different between the various diets.[49] Moreover, a cis-PUFA diet did not affect apoA-I FCR[50] and both FCR and PR decreased after low fat consumption compared with high cis-MUFA consumption.[51] In contrast, Labonte et al. have reported that replacing 13 energy % of carbohydrates with cis-MUFA decreased apoA-I FCR with no change in apoA-I PR (Table 3).[52] The different results between these two studies [51,52] may have been due to the significant weight loss in the study of Desroches et al., which may have confounded to some extent the effect of MUFA on apoA-I kinetics.

Table 2 *Effect of boiled and filtered coffee, caffeine and tea on fasting apoA-I concentrations*

First author, Year	Food component / product	Study design and duration	Participants	Intake	Effects
Aro et al. (1987)[42]	Boiled vs. filtered coffee vs. tea	RCT cross over 4 weeks	42 hypercholesterolemic subjects	8 cups/day	- No differences in serum apoA-I concentrations
Aro et al. (1990)[41]	Boiled vs. filtered coffee	RCT cross over 4 weeks	41 healthy subjects	2-14 cups/day	- No differences in serum apoA-I concentrations
Van Dusseldorp et al. (1991)[46]	Filtered vs. unfiltered coffee vs. no coffee	RCT parallel 79 days	64 healthy subjects	6 cups/day	- No differences in serum apoA-I concentrations
Burr et al. (1989)[43]	Decaffeinated vs. no coffee	RCT cross over 4 weeks	54 healthy subjects	>5 cups/day	- No differences in plasma apoA-I concentrations
Davies et al. (2003)[44]	Black tea vs. caffeine vs. caffeine free placebo	RCT cross 3 weeks over	15 mildly hypercholesterolemic subjects	5 cups/day	- No differences in plasma apoA-I concentrations
Mozaffarik-Khosravi et al. (2009)[45]	Sour tea vs. black tea	RCT parallel 1 month	53 patients with diabetes mellitus type 2	2 cups/day	- No differences in serum apoA-I concentrations

Fish and fish-fatty acids

Most studies investigating the effects of omega-3 fatty acids from fatty fish, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), did not observe any differences in fasting and postprandial apoA-I concentrations.[53-75] However, in two studies, all in healthy men, fasting apoA-I concentrations decreased after fish oil supplementation. The first study showed lower apoA-I concentrations after pullock oil (rich in EPA) and salmon oil (rich in DHA), but not after tuna oil (rich in DHA) consumption as compared with butter.[76] The second study found lower apoA-I concentrations after EPA oil supplementation compared with DHA oil supplementation.[77] On the other hand, one study found an increase in fasting apoA-I concentrations after a diet high in fish-fatty acids compared with a diet low in fish-fatty acids, in which diets were matched for total fat (Table 4).[78]

Five studies have investigated the effects of fish on fasting apoA-I concentrations. In one study, fatty fish (salmon, rainbow trout, Baltic herring, whitefish, vendace and tuna) consumption increased apoA-I concentrations compared with lean fish (pike, pike-perch, perch, saithe and cod) consumption. However, it did not change apoA-I concentrations as compared with lean meat (beef and pork) consumption.[79] The other three studies did not find differences in apoA-I concentrations after fish consumption, of which two compared fatty fish with lean meat[80,81] and one compared prawns with crab.[82] A limitation of the study of Lindqvist et al. is that participants consumed in total 35 energy % of fat in the herring period and only 10 energy % of fat in the meat period,[80] which may have affected apoA-I concentrations. Comparisons between fish and meat consumption are probably not confounded by differences in the intake of the source of protein, as suggested by Gascon et al. In that study, the effects of proteins in lean fish (cod, sole, pollack, and haddock) were compared with those of animal protein (lean beef, pork, veal, eggs, skimmed milk and milk products). No differences on fasting apoA-I concentrations were found (Table 4).[83]

Fibers

Studies comparing the effects of oat germ – low in fiber - with those of wheat germ – high in fiber- consumption did not find any differences in fasting apoA-I concentrations.[84-89] In four of these studies, it was explicitly reported that the macronutrient composition of the experimental diets was comparable.[84,85,87,88] Mekki et al. observed that a high-fiber diet did not change fasting apoA-I concentrations as compared with a low-fiber diet.[90] On the other hand, decreased fasting apoA-I concentrations were found after a high β-glucan and psyllium diet as compared with a low fat, low cholesterol control diet.[91] The water-soluble fiber arabinoxylan also decreased fasting apoA-I concentrations as compared with the control diet, which had a similar macronutrient composition.[92] Furthermore, no differences in

Table 3 Meta-analysis showing the effects of fatty acids on fasting apoA-I concentrations and studies showing effects of fatty acids on apoA-I kinetics

First author, Year	Food component /product	Study design and duration	Participants	Intake	Effect
Mensink (2016)[47]	Replacement of carbohydrates (carbs) for SFA, MUFA or PUFA	Meta-analysis of 42 studies till Dec 2013: Daily controlled RCT parallel and cross over > 13 days	Healthy subjects	1% of dietary energy	- 8.4 mg/dL (95% CI 6.4 - 10.5) ↓ in fasting apoA-I concentrations replacing carbs with SFA - 5.5 mg/dL (95% CI 3.7 - 7.3) ↓ in fasting apoA-I concentrations replacing carbs with MUFA - 2.3 mg/dL (95% CI 0.1 - 4.6) ↓ in fasting apoA-I concentrations replacing carbs with PUFA
Mensink (2016)[47]	Replacement of carbs for lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) or stearic acid (C18:0)	Meta-analysis of 34 studies till Dec 2013: Daily controlled RCT parallel and cross over > 13 days	Healthy subjects	1% of dietary energy	- 19.2 mg/dL (95% CI 14.6 - 12.7) ↓ in fasting apoA-I concentrations replacing carbs with lauric acid - 8.8 mg/dL (95% CI 0.5 - 13.1) ↓ in fasting apoA-I concentrations replacing carbs with myristic acid - 6.5 mg/dL (95% CI 3.8 - 9.3) ↓ in fasting apoA-I concentrations replacing carbs with palmitic acid - No difference in apoA-I concentrations replacing carbs with stearic acid
Brouwers (2016)[48]	Replacement of trans fatty acids (TFA) for carbs	Meta-analysis of 10 studies till Sep 2014: Daily controlled RCT parallel and cross over > 13 days	Healthy subjects	1% of dietary energy	- 3.3 mg/dL (95% CI 4.7 - 1.9) ↓ in fasting apoA-I concentrations replacing carbs with TFA - 2.6 mg/dL (95% CI 1.4 - 3.9) ↓ in fasting apoA-I concentrations replacing TFA with SFA - No difference in apoA-I concentrations replacing TFA with MUFA - 1.7 mg/dL (95% CI -2.8 - -0.6) ↑ in fasting apoA-I concentrations replacing TFA with PUFA
Ginsberg et al. (1994)[50]	Average American diet vs PUFA enriched diet	RCT parallel 6 weeks	21 healthy men	MUFA: 14 vs. 8 energy% PUFA: 7 vs. 13 energy%	- No difference in apoA-I FCR

Table 3 *Meta-analysis showing the effects of fatty acids on fasting apoA-I concentrations and studies showing effects of fatty acids on apoA-I kinetics (continued)*

First author, Year	Food component /product	Study design and duration	Participants	Intake	Effect
Desroches et al. (2004)[51]	Low fat diet vs. high MUFA diet	RCT parallel 6-7 weeks	18 healthy men	Fat: 25.8 vs. 40.1 energy% MUFA: 13.3 vs. 22.5 energy%	- 31% ↑ PR after low fat compared with high MUFA diet - 22% ↑ FCR after low fat compared with high MUFA diet
Matthan et al. (2004)[49]	Soybean oil (PUFA) vs. margarine (TSA) vs. butter (SFA)	RCT cross over 5 weeks	8 hyper-cholesterolemic women	2/3 of the total fat intake	- 11% ↓ FCR after margarine compared with butter - No difference in PR between the diets
Labonte et al. (2013)[52]	Carbohydrates vs. MUFA	RCT parallel 4 weeks	16 dyslipidemic subjects	13 energy%	- 5.6% ↓ in FCR after carbohydrate compared with MUFA consumption - No difference in PR between the diets

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TFA: trans fatty acids, PR: production rate, FCR: fractional catabolic rate

Table 4 *Effect of fish oil or fish on apoA-I concentration*

First author, Year	Food component/ Product	Study design and duration	Participants	Intake	Effect
Schectman et al. (1988)[55]	Low vs. high fish oil vs. safflower oil capsules	RCT cross over 1 month	13 patients with non-insulin-dependent diabetes mellitus type 2	4.0 vs. 7.5 g omega-3/ day, 12 g safflower oil/day	- No differences in fasting plasma apoA-I concentrations
Wilt et al. (1989)[56]	Fish oil vs. safflower oil capsule	RCT parallel 12 weeks	38 healthy men	20 g/day	- No differences in fasting plasma apoA-I concentrations
Childs et al. (1990)[76]	Pullock oil (EPA) vs. tuna (DHA) vs. salmon (DHA) vs. butter capsule	RCT cross over 3 weeks	8 healthy men	EPA: 11.7, 5.4 and 6.1 g/day DHA: 3.1, 15.5, 7.7 g/day	- 22.0%, 14.0%, 0.0% ↑ in fasting plasma apoA-I concentrations comparing pullock oil, salmon oil, or tuna oil with control, respectively
DeLany et al. (1990)[57]	Low vs. high fish oil vs. margarine (similar macronutrient composition)	RCT parallel 5 weeks	15 healthy men	0, 5, 20 g fish oil/ day with 0, 2, 8 g omega-3/day	- No differences in fasting serum apoA-I concentrations
Levinson et al. (1990)[58]	Fish oil vs. vegetable oil capsule	RCT parallel 6 weeks	16 mild hypertensive patients	50 g oil/day, 9 g EPA, 6 g DHA	- No differences in fasting serum apoA-I concentrations
Mori et al. (1990)[59]	Fish oil vs. no fish oil	RCT parallel 3 weeks	22 insulin-dependent diabetic men	2.7 g EPA and 1.7 g DHA/day	- No differences in fasting serum apoA-I concentrations
Bonaa et al. (1992)[60]	EPA, DHA oil vs. corn oil capsule	RCT parallel 10 weeks	156 healthy subjects	5.1 g/day	- No differences in fasting serum apoA-I concentrations
Richter et al. (1992)[54]	Omega-3 vs. omega-6 capsule	RCT cross over 3 weeks	26 healthy men	5.0 g/day	- No differences in fasting plasma apoA-I concentrations
Tato et al. (1993)[61]	EPA and DHA vs. olive oil capsules	RCT cross over 4 weeks	9 patients with familial hyperlipidemia	3.0 and 4.5 g EPA and DHA/day	- No differences in fasting serum apoA-I concentrations
Zampelas et al. (1994)[62]	SFA oil vs. corn oil vs. fish oil capsule	RCT cross over 1 day	12 healthy men	40 g/day	- No differences in postprandial serum apoA-I concentrations
Eritsland et al. (1995)[63]	Fish oil capsule vs. no capsule	RCT parallel 9 months	511 patients with coronary artery disease	4g/day: 3.4 g EPA and DHA	- No differences in fasting serum apoA-I concentrations

Table 4 *Effect of fish oil or fish on apoA-I concentration (continued)*

First author, Year	Food component/ Product	Study design and duration	Participants	Intake	Effect
Herrmann et al. (1995)[64]	Omega-3 vs. rapeseed oil capsule	RCT parallel 4 weeks	53 patients with coronary artery disease	8.5 g/day	- No differences in fasting plasma apoA-I concentrations
Hamazaki et al. (1996)[65]	DHA vs. control oil capsule	RCT parallel 13 weeks	24 healthy subjects	1.5-1.8 g/day	- No differences in fasting serum apoA-I concentrations
Grimsgaard et al. (1997)[77]	EPA vs. DHA vs. corn oil capsule	RCT parallel 7 weeks	234 healthy men	EPA: 3.8 g/day, DHA: 3.6 g/day, Corn oil: 4.0 g/day	- 5.0% ↑ in fasting serum apoA-I concentrations comparing EPA with corn oil * - No differences in fasting serum apoA-I concentrations comparing DHA with corn oil *
Sorensen et al. (1998)[66]	Fish oil vs. sunflower oil margarine	RCT parallel 4 weeks	47 healthy subjects	4.0 g/day	- No differences in fasting plasma apoA-I concentrations
Buckley et al. (2004)[67]	EPA vs. DHA vs. olive oil capsules	RCT parallel 4 weeks	42 healthy subjects	EPA: 4.8 g/day DHA: 4.9 g/day	- No differences in fasting plasma apoA-I concentrations
Calabresi et al. (2004)[68]	Omega-3 vs. placebo capsule	RCT cross over 8 weeks	14 patients with familial hyperlipidemia	EPA: 1.88 g/day, DHA: 1.48 g/day	- No differences in fasting plasma apoA-I concentrations
Shidfar et al. (2003)[71]	Omega-3 vs. placebo	RCT parallel 10 weeks	68 hyperlipidemic patients	1 g/day	- No differences in fasting serum apoA-I concentrations
Li et al. (2004)[78]	High vs. low fish fatty acids diet	RCT parallel 24 weeks	22 healthy subjects	30 energy% of fat	- 14.0% ↓ in fasting plasma apoA-I concentrations comparing high with low fish fatty acids consumption
Goyens et al. (2006)[69]	ALA vs. EPA and DHA capsule	RCT parallel 6 weeks	37 elderly healthy subjects	ALA 6.8 g/day, EPA and DHA: 1.05 + 0.55 g/day	- No differences in fasting serum apoA-I concentrations
De Roos et al. (2008)[70]	Fish oil vs. high oleic sunflower oil capsule	RCT parallel 6 weeks	81 healthy subjects	3.5 g/day: 700 mg EPA, 560 mg DHA	- No differences in fasting serum apoA-I concentrations
Shidfar et al. (2008)[53]	Omega-3 vs. 300mg SFA, 100mg MUFA, 600mg linoleic acid capsule	RCT parallel 10 weeks	50 patients with diabetes mellitus type 2	2 g/day: 520 mg EPA, 480 mg DHA	- No differences in fasting serum apoA-I concentrations

Table 4 Effect of fish oil or fish on apoA-I concentration (continued)

First author, Year	Food component/ Product	Study design and duration	Participants	Intake	Effect
Maki et al. (2011)[72]	Omega-3 vs. soy oil	RCT cross over 6 weeks	31 patients with primary, isolated hypercholesterolemia	4 g/day	- No differences in fasting serum apoA-I concentrations
Ooi et al. (2012)[73]	Therapeutic lifestyle change diet low vs. high in fish (capsule)	RCT parallel 24 weeks	20 healthy subjects	EPA and DHA: 1.23 g/ day vs. 0.27 g/day	- No differences in fasting plasma apoA-I concentrations
Song et al. (2013)[74]	High omega-3, low omega-6 vs. low omega-3, high omega-6 fatty acid diet	RCT cross over 1 day	8 healthy and 8 hyper-triacylglycerolemic subjects	0.97 vs 8.80 n-6/n-3 ratio	- No differences in postprandial serum apoA-I concentrations
Oliveira et al. (2014)[75]	Fish oil vs. soya oil supplements	RCT parallel 24 weeks	83 HIV-infected subjects on antiretroviral therapy	3 g/day: 540mg EPA, 360mg DHA	- No differences in postprandial serum apoA-I concentrations
Van Houwelingen et al. (1990)[81]	Mackerel vs. lean meat	RCT cross over 6 weeks	84 healthy male subjects	135 g/day	- No differences in fasting serum apoA-I concentrations
Gascon et al. (1996)[83]	Lean fish (cod, sole, pollack, haddock) vs. animal protein (lean beef, pork, veal, eggs, skimmed milk, milk products)	RCT cross over 4 weeks	14 premenopausal healthy women	69-71 energy % protein	- No differences in lean fish on fasting plasma apoA-I concentrations
Lindqvist et al. (2007)[80]	Herring vs. lean meat (pork and chicken)	RCT cross over 4 weeks	13 healthy obese subjects	50 g/day, 5 days/week	- No differences in fasting plasma apoA-I concentrations
Erkkila et al. (2008)[79]	Fatty (salmon, rainbow trout, Baltic herring, whitefish, vendace, tuna) vs. lean fish (pike, pike-perch, perch, saithe, cod) vs. lean meat (beef, pork)	RCT parallel 8 weeks	33 patients with coronary heart disease	100-150 g fish, 4 meals/week	- 7.1 and 9.1 % ↑ in fasting serum apoA-I concentrations comparing lean fish with lean meat or fatty fish consumption *
Isherwood et al. (2010)[82]	Prawns vs. crab sticks	RCT cross over 12 weeks	23 healthy males	225 g/day	- No differences in fasting plasma apoA-I concentrations

** Percentages calculated from the mean values, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, SFA: saturated fatty acids, ALA: alpha lipoic acid*

fasting apoA-I concentrations were observed between the soluble and insoluble forms of P. ovate.[93] The water-soluble fiber β-glucan did not affect fasting apoA-I concentrations.[94] Furthermore, wheat germ consumption increased fasting apoA-I concentrations compared with flaxseed consumption (Table 5).[95]

Nuts

In one short-term study, walnut consumption significantly increased fasting serum apoA-I concentrations,[96] but these effects were not found in two longer-term studies.[97,98] Almond consumption did also not affect fasting apoA-I concentrations.[99,100] Likewise, hazelnuts[101,102] and pistachio nuts did not change fasting apoA-I concentrations.[103] A limitation of some of the studies is, that not all experimental diets were matched for differences in fat and fatty acid composition. In some of these studies, the diets containing nuts provided more energy from fat than the control diets.[98-101] Furthermore, the nut diets were sometimes also lower in SFA and higher in PUFA than the control diets.[98,100] Although these differences in nutrient intakes are inherent to consuming more nuts, it is not likely that the effects observed are due to minor component in nuts, since fatty acids increase apoA-I concentrations as compared with carbohydrates.[47] However, most other studies that used a control diet with similar fat and fatty acid composition did also not find any effects of the consumption of nuts on apoA-I concentrations (Table 6).[97,102,103]

Plant sterols and stanols

Most studies examining the effects of plant sterols on serum lipids did not demonstrate an effect of plant sterols on fasting apoA-I concentrations.[104-111] In one study, comparing olive oil, olive oil with plant sterol esters, and sunflower oil with plant sterol esters, fasting apoA-I concentrations increased when plant sterol esters where consumed together with olive oil, but apoA-I concentrations were comparable during the other two interventions.[112] Furthermore, one study showed an increase in fasting apoA-I concentrations comparing 3 months of prudent diet consumption (National Cholesterol Education Program) with added plant sterols, with prudent diet consumption alone.[105] One study examined the effects of plant stanols on fasting apoA-I concentrations and found increased apoA-I concentrations comparing 6 weeks of sitostanol consumption with no sitostanol consumption.[113] Finally, no changes in apoA-I PR and FCR were found after plant sterol or stanol consumption (Table 7). [107,113]

Soy proteins or isoflavones isolated from soy

Studies investigating the effects of soy protein on fasting apoA-I concentrations showed inconsistent outcomes. Eight studies using different amounts of soy protein for 3 weeks till 3 months did not find changes in fasting apoA-I concentrations.[114-122]

Table 5 *Effect of fiber on fasting apoA-I concentrations*

First author, Year	Food component/product	Study design and duration	Participants	Intake	Effect
Anderson et al. (1991)[84]	Oat vs. wheat bran diet (similar macronutrient composition)	RCT parallel 3 weeks	20 hypercholesterolemic men	14 g/day	- No differences in serum apoA-I concentrations
Cara et al. (1992)[85]	Oat vs. rice vs. wheat bran vs. wheat germ (similar macronutrient composition)	RCT cross over 1 day	6 healthy subjects	10 g as oat, rice, wheat bran vs. 4.2 g as wheat germ	- No differences in serum apoA-I concentrations
Kashtan et al. (1992)[86]	Oat vs. wheat bran supplemented food	RCT parallel 2 weeks	32 subjects with a history of polypectomy and 32 healthy subjects	6.8 g/1000 kcal/day	- No differences in serum apoA-I concentrations
Stewart et al. (1992)[87]	Oat bran vs. control diet (similar macronutrient composition)	RCT cross over 6 weeks	24 hypercholesterolemic subjects	50 g/day	- No differences in serum apoA-I concentrations
Uusitupa et al. (1992)[88]	Oat vs. wheat bran diet (similar macronutrient composition)	RCT parallel 8 weeks	36 hypercholesterolemic subjects	10.3 g/day	- No differences in serum apoA-I concentrations
Zhang et al. (1992)[89]	Oat vs. wheat bran	RCT cross over 3 weeks	9 subjects with ileostomies	4.9 vs 29.0 g/day	- No differences in plasma apoA-I concentrations
Mekki et al. (1997)[90]	High fiber diet vs. low fiber diet	RCT parallel 4 weeks	31 mildly hypercholesterolemic subjects	35 g/day	- No differences in plasma apoA-I concentrations
Jenkins et al. (2002)[91]	Low fat, low cholesterol diet high vs. low in β-glucan or psyllium fiber (similar macronutrient composition)	RCT cross over 4 weeks	68 hyperlipidemic subjects	8 g/day	- 1.3% ↑ in serum apoA-I concentrations comparing the high with the low fibers*

Table 5 Effect of fiber on fasting apoA-I concentrations (continued)

First author, Year	Food component/product	Study design and duration	Participants	Intake	Effect
Garcia et al. (2006)[92]	Arabinoxylan supplement vs. placebo	RCT cross over 6 weeks	11 patients with impaired glucose tolerance	15 g/day	- 4.0% ↑ in serum apoA-I concentrations *
Sola et al. (2007)[93]	Low SFA diet supplemented with P. ovata husk vs. P. ovata seeds	RCT cross over 8 week	28 men with CVD	10.5 g/day	- No differences in fasting plasma apoA-I concentrations
Rondanelli et al. (2008)[94]	β-glucan vs. rice bran supplemented food	RCT cross over 4 weeks	24 mildly hypercholesterolemic men	15 vs. 30 g/day	- No differences in serum apoA-I concentrations
Dodin et al. (2008)[95]	Flaxseed vs. wheat germ	RCT parallel 12 months	199 healthy postmenopausal women	40 g/day	- 4.0% ↑ in serum apoA-I concentrations comparing flaxseed with wheat germ *

** Percentages calculated from the mean values, SFA: saturated fatty acids*

Table 6 *Effect of different nuts on fasting apoA-I concentrations*

First author, Year	Food component/product	Study design and duration	Participants	Intake	Effects
Munoz et al. (2001)[97]	Walnuts vs. mediterranean cholesterol-lowering diet (similar macronutrient composition)	RCT cross over 6 weeks	10 hypercholesterolemic men	41-56 g/day	- No differences in serum apoA-I concentrations
Rajaram et al. (2009)[98]	Walnut vs. control diet	RCT cross over 4 weeks	25 mildly hyperlipidemic subjects	42.5 g/day	- No differences in serum apoA-I concentrations
Aronis et al. (2012)[96]	Walnut vs. control diet (similar macronutrient composition)	RCT cross over 4 days	15 patients with metabolic syndrome	48 g/day	- 8.1% ↓ in serum apoA-I concentrations comparing walnut with control diet *
Sabate et al. (2003)[100]	Diet without vs. low vs. high in almonds	RCT cross over 4 weeks	25 healthy subjects	0, 10, 20 energy %	- No differences in serum apoA-I concentrations
Li et al. (2011)[99]	Almond vs. NCEP[1] diet	RCT cross over 12 weeks	20 patients with diabetes mellitus type 2	60 g/day	- No differences in plasma apoA-I concentrations
Mercanligil et al. (2007)[101]	Hazelnut vs. low-fat, low-cholesterol high-carbohydrate diet	Period 1 control, period 2 intervention 4 weeks	15 hypercholesterolemic men	40 g/day	- No differences in plasma apoA-I concentrations
Tey et al. (2011)[102]	Ground vs. sliced vs. whole hazelnuts	RCT cross over 4 weeks	48 mildly hypercholesterolemic subjects	30 g/day	- No differences in plasma apoA-I concentrations
Sheridan et al. (2007)[103]	Pistachio vs. control diet (similar macronutrient composition)	RCT cross over 4 weeks	15 mildly hypercholesterolemic subjects	56-85 g/day	- No differences in serum apoA-I concentrations

** Percentages calculated from the mean values, [1] NCEP: National Cholesterol Education Program step II*

Table 7 *Effect of plant sterols on fasting apoA-I concentrations and apoA-I kinetics*

First author, Year	Food component /product	Study design and duration	Participants	Intake	Effects on
Gylling et al. (1994)[113]	Sitostanol vs. control margarine	RCT cross over 6 weeks	11 hypercholesterolaemic diabetic men	3.0 g/day	- 4.3% in fasting serum apoA-I concentrations * - No difference in apoA-I FCR
Temme et al. (2001)[110]	Plant sterols enriched vs. non plant sterol enriched margarine	RCT cross over 4 weeks	42 mildly hypercholesterolemic subjects	2.0 g/day	- No differences in serum apoA-I concentrations
Amundsen et al. (2002)[104]	Plant sterol esters vs. control with similar fatty acid composition	RCT cross over 8 weeks	38 children with familial hypercholesterolemia	1.6 g/day	- No differences in plasma apoA-I concentrations
Chan et al. (2007)[112]	Olive oil vs. sunflower oil with plant sterols vs. olive oil with plant sterols margarine	RCT cross over 4 weeks	21 moderately overweight, hypercholesterolemic subject	70% of total fat in the diet 1.7 g plant sterols/ day	- 0.8% ↓ in fasting plasma apoA-I concentrations comparing olive oil with plant sterols with olive oil alone or sunflower oil with plant sterols *
Madsen et al. (2007)[106]	Plant sterols vs. control with similar fatty acid composition	RCT cross over 4 weeks	46 mildly hypercholesterolemic subjects	2.3 g/day	- No differences in serum apoA-I concentrations
Ooi et al. (2007)[107]	Plant sterols enriched vs. non plant sterol enriched margarine and cereals	RCT cross over 4 weeks	9 patients with the metabolic syndrome	2.0 g/day	- No differences in plasma apoA-I concentrations - No effect on apoA-I PR - No effect on apoA-I FCR
Hernandez-Mijares et al. (2010)[105]	Healthy diet (NCEP)[1] vs. healthy diet with plant sterols vs. normal diet with plant sterols	RCT parallel 3 months	84 mildly hypercholesterolemic subjects	2.0 g/day	- 4.0% ↓ in serum apoA-I concentration comparing prudent diet with plant sterols with prudent diet alone, or normal diet with plant sterols *
Soderholm et al. (2010)[109]	Rye bread with low vs. high vs. no plant sterols	RCT parallel 2 weeks	68 healthy subjects	2.0 vs. 4.0 g/day	- No differences in serum apoA-I concentrations

Table 7 *Effect of plant sterols on fasting apoA-I concentrations and apoA-I kinetics (continued)*

First author, Year	Food component /product	Study design and duration	Participants	Intake	Effects on
Gagliardi et al. (2010)[111]	Plant sterol margarines vs. no-trans FA margarine vs. butter	RCT parallel 5 weeks	53 subjects with metabolic syndrome	2.4 g/day	- No differences in plasma apoA-I concentrations
Sialvera et al. (2012)[108]	Yogurt beverage with vs. without phytosterol	RCT parallel 2 months	108 patients with the metabolic syndrome	4.0 g/day	- No differences in plasma apoA-I concentrations

** Percentages calculated from the mean values, [1] NCEP: National Cholesterol Education Program*

On the other hand, in one study products containing soy protein increased fasting apoA-I concentrations as compared with products containing casein,[123] while in another study products with soy protein decreased fasting apoA-I as compared with products containing casein.[124] Furthermore, two studies found different effects of various soy products on fasting apoA-I concentrations.[125,126] Soymilk increased apoA-I concentrations as compared with soy nuts and soy flour, but no differences were found as compared with animal protein.[126] Soy nut and soy protein consumption increased apoA-I concentrations as compared with the control group without soy.[125] Two studies have investigated the effects of isoflavones isolated from soy on apoA-I concentrations and showed no effect on fasting [127,128] and postprandial apoA-I concentrations (Table 8).[127]

Others

Many other products and food components have been studied for their effects on apoA-I concentrations. In most of these studies, which included eggs,[129] dried garlic,[130,131] beta-caroteen,[132] phytochemicals with cytochrome P-450-inducing activity,[133] magnesium,[134] eggplant,[135] dry beans,[136] kiwifruits[137] and polyphenols,[138] no effects on fasting apoA-I concentrations were observed. In addition, sphingolipids did not change postprandial apoA-I concentrations.[139] On the other hand, red grape juice,[140] a mixture of citrus flavonoids and tocotrienols,[141] vitamin D supplementation,[142,143] vitamin D plus calcium supplementation,[143] theobromine,[144] orange juice,[145] and a high dose of grape pomace and omija fruit,[146] all increased fasting apoA-I concentrations (Table 9).

Table 8 Effect of soy protein or isoflavone in soy on apoA-I concentrations

First author, Year	Food component/ product	Study design and duration	Participants	Intake	Effect
Bakhit et al. (1994)[114]	Soybean protein with or without soybean fiber	RCT cross over 4 weeks	21 hypercholesterolemic men	25 g/day	- No differences in fasting plasma apoA-I concentrations
Kurowska et al. (1997)[119]	Soy protein vs. milk protein	RCT cross over 4 weeks	34 hypercholesterolemic subjects	2% of daily intake	- No differences in fasting plasma apoA-I concentrations
Nilausen et al. (1998)[123]	Soy protein vs. casein (similar macronutrient composition)	RCT cross over 1 month	9 healthy men	154 g/day	- 10.7% ↓ in fasting plasma apoA-I concentrations *
Jenkins et al. (2000)[118]	Soy incorporated into breakfast cereals vs. no soy	RCT cross over 3 weeks	25 hyperlipidemic subjects	36 g/day	- No differences in fasting serum apoA-I concentrations
Chen et al. (2006)[115]	Soy protein vs. milk protein	RCT parallel 3 months	26 patients undergoing hypercholesterolaemic haemodialysis	30 g/day	- No differences in fasting serum apoA-I concentrations
McVeigh et al. (2006)[120]	Soy protein varying in isoflavone content	RCT cross over 57 days	35 healthy young men	1.64-61.7 mg isoflavone/day	- No differences in fasting serum apoA-I concentrations
Pipe et al. (2009)[121]	Soy protein isolate vs. milk protein isolate	RCT cross over 57 days	29 patients with diabetes mellitus type 2	80 g/day	- No differences in fasting serum apoA-I concentrations
Campbell et al. (2010)[124]	Soy protein products vs. casein products	RCT parallel 1 year	62 moderately hyper-cholesterolemic women	25 g/day	- 8.5 % ↑in fasting serum apoA-I concentrations *
Tabibi et al. (2010)[122]	Soy protein vs. no soy protein	RCT parallel 8 weeks	40 peritoneal dialysis patients	28 g/day	- No differences in fasting serum apoA-I concentrations
Jenkins et al. (2002)[117]	High- vs. low-isoflavone soy protein	RCT cross over 1 months	41 hyperlipidemic subjects	Soy: 50-52 g/day Isoflavones: 73 vs. 10 mg/day	- No differences in fasting serum apoA-I concentrations

Table 8 Effect of soy protein or isoflavone in soy on apoA-I concentrations (continued)

First author, Year	Food component/ product	Study design and duration	Participants	Intake	Effect
Cicero et al. (2002)[116]	Soy proteins supplemented with isolated b-sitosterol vs. no soy-protein	RCT parallel 40 days	20 moderately hyper-cholesterolemic subjects	10 g/day	- No differences in fasting plasma apoA-I concentrations
Matthan et al. (2007)[126]	Different sources of soy protein vs. animal protein	RCT cross over 6 weeks	28 hypercholesterolemic subjects	6.8-7.5 energy%/ day	- 2.0% ↓ in fasting plasma apoA-I concentrations comparing soy-milk with soybean and soy-flour - No differences in fasting plasma apoA-I concentrations comparing soy with animal protein
Bakhtiary et al. (2012)[125]	Soy protein vs. soy nuts vs. no soy	RCT parallel 3 months	75 women with the metabolic syndrome	35 g/day	- 18.8% and 25.0% ↓ in fasting serum apoA-I concentrations comparing soy-protein or soy-nut with control, respectively *
Wangen et al. (2001)[128]	No vs. low vs. high soy isoflavone	RCT cross over 3 months	18 mildly hyper-cholesterolemic women	7.1, 65, 132 mg/day	- No differences in fasting plasma apoA-I concentrations
Santo et al. (2010)[127]	Milk-protein vs. isoflavone-poor soy vs. isoflavone-rich soy	RCT cross over 28 days	30 healthy young men	25 g protein/day	- No differences in fasting and postprandial plasma apoA-I concentrations

** Percentages calculated from the mean values*

Table 9 Effect of other food components or products on apoA-I concentrations

First author, Year	Food component / product	Study design and duration	Participants	Intake	Effects
Sacks et al. (1984)[129]	Eggs vs. no foods containing eggs	RCT cross over 3 weeks	17 healthy subjects	400 kcal/day	- No differences in fasting plasma apoA-I concentrations
Luley et al. (1986)[130]	Dried garlic vs. control	RCT cross over 6 weeks	- 34 hyperlipemic patients	3x198 mg/day	- Both no differences in fasting serum apoA-I concentrations
Luley et al. (1986)[130]	Dried garlic vs. control	RCT cross over 6 weeks	- 51 hyperlipemic patients	3x450 mg/day	- Both no differences in fasting serum apoA-I concentrations
Hughes et al. (1994)[132]	Beta-carotene vs. wheat germ oil capsules	RCT parallel 30 days	59 hyperlipidemic patients 36 healthy subjects	300 mg/day	- No differences in fasting serum apoA-I concentrations
Nanjee et al. (1996)[133]	Glucosinolate free vegetable vs. Brussels sprouts	RCT parallel 3 weeks	10 healthy men	300 g/day	- No differences in fasting plasma apoA-I concentrations
Nanjee et al. (1996)[133]	Eugenol vs. placebo capsule	RCT parallel 3 weeks	10 healthy men	150 mg/day	- No differences in fasting plasma apoA-I concentrations
Neil et al. (1996)[131]	Dried garlic vs. placebo powder	RCT parallel 6 months	115 hypercho- lesterolemic subjects	900 mg/day	- No differences in fasting serum apoA-I concentrations
Itoh et al. (1997)[134]	Magnesium vs. placebo supplement	RCT parallel 4 weeks	33 healthy subjects	411-548 mg/day	- No differences in fasting serum apoA-I concentrations
Guimaraes et al. (2000)[135]	Eggplant vs. placebo powder	RCT parallel 5 weeks	38 hypercho- lesterolemic subjects	12 g powder/day, corresponded with 100 g eggplant/day	- No differences in fasting serum apoA-I concentrations
Oosthuizen et al. (2000)[136]	Dry beans vs. no beans	RCT cross over 4 weeks	22 hyperlipidemic patients	110 g/day	- No differences in fasting serum apoA-I concentrations
Gammon et al. (2012)[137]	Healthy diet with vs. without green kiwifruits	RCT cross over 4 weeks	85 hypercholesterolemic men	2 kiwifruits/day	- No differences in fasting serum apoA-I concentrations

Table 9 Effect of other food components or products on apoA-I concentrations (continued)

First author, Year	Food component / product	Study design and duration	Participants	Intake	Effects
Mullan et al. (2016)[138]	Polyphenols vs. control	RCT parallel 4 weeks	20 healthy overweight or obese subjects	250 ml (361 mg) polyphenols + 120 mg vitamin C	- No differences in fasting plasma apoA-I concentrations
Ohlsson et al. (2010)[139]	Sphingolipids vs. placebo	RCT parallel 1 day	18 healthy men	40 g high fat meal 975 mg milk sphingolipids	- No differences in postprandial plasma apoA-I concentrations
Castilla et al. (2006)[140]	Red grape juice vs. no juice	RCT parallel 2 weeks	26 hemodialysis patients 12 hemodialysis control patients 15 healthy subjects	100 ml/day	- 13.2% ↓ in fasting plasma apoA-I concentrations comparing juice with no juice consumption in hemodialysis patients * - 63.2% ↓ in fasting plasma apoA-I concentrations comparing juice consumption in healthy subjects with no juice consumption in hemodialysis patients *
Roza et al. (2007)[141]	Citrus flavonoids with tocotrienols vs. placebo	RCT parallel 12 weeks	120 hypercholesterolemic subjects	270 mg citrus flavonoids + 30 mg tocotrienols/day	- 5.0% ↓ in fasting plasma apoA-I concentrations
Salehpour et al. (2012)[142]	Vitamin D3 vs. control supplement	RCT parallel 12 weeks	77 healthy overweight or obese subjects	25 mg/day	- 9.2% ↓ in fasting serum apoA-I concentrations *
Heravifard et al. (2013)[143]	Vitamin D vs. calcium and vitamin D vs. control	RCT parallel 12 weeks	90 patients with diabetes mellitus type 2	150 mg calcium vs. 150 mg calcium and 500 IU vitamin D vs. 250 mg calcium and 500 IU vitamin D	- 18% ↓ in fasting serum apoA-I concentrations comparing vitamin D with control * - 16% ↓ in fasting serum apoA-I concentrations comparing vitamin D with calcium with control *
Neufingerl et al. (2013)[144]	Theobromine vs placebo	RCT parallel 4 weeks	152 healthy subjects	0, 150, 850, 1000 mg/day	- 7.6% ↓ in fasting serum apoA-I concentrations comparing 850mg theobromine with placebo *

Table 9 *Effect of other food components or products on apoA-I concentrations (continued)*

First author, Year	Food component / product	Study design and duration	Participants	Intake	Effects
Constans et al. (2015)[145]	Orange juice vs. control	RCT cross over 4 weeks	25 male subjects with 2 CVD risk factors	3x daily 200 ml	- 6.2% ↓ in fasting plasma apoA-I concentrations comparing orange juice with placebo *
Han et al. (2016)[146]	Low vs. high dose of grape pomace and omija fruit vs. control	RCT parallel 10 weeks	76 healthy overweight or obese subjects	342.5 vs. 685.0 mg grape pomace/day and 57.5 vs. 115.0 mg omija/day	- 10% ↓ in fasting plasma apoA-I concentrations after the high dose compared with control * - No difference in fasting plasma apoA-I concentrations after the low dose compared with control

** Percentages calculated from the mean values*

Pharmacological approaches targeting apoA-I metabolism

Although not always specifically developed for this purpose, several well-known drugs like statins[6,147] and CETP inhibitors[148-151] affect serum apoA-I concentrations. However, since this review focuses on novel strategies to increase serum apoA-I concentrations, we here describe only approaches, that are currently in development, and are specifically designed to target apoA-I metabolism. Potentially relevant studies published before January 2017 were identified by a systematic search of the database PubMed (www.ncbi.nlm.nih.gov). The following search terms were used to search in titles and abstracts: (Pharmacological AND approaches AND apoA-I). First, all abstracts were screened and the pharmacological approaches were divided into three categories; apoA-I mimetics, apoA-I infusions, and others. Second, a new search was performed with the search terms: (apoA-I mimetics AND apoA-I infusions AND RVX-208 AND LCAT infusion) to select all studies published before January 2017 that investigate apoA-I mimetics, apoA-I infusions, and RVX-208.

ApoA-I mimetics

ApoA-I mimetics are small amphipathic peptides that resemble apoA-I in biological function and structure.[152] These mimetics are not the intact apoA-I protein, but small fragments of the protein with certain biological functions. These small peptides can be given orally or can be infused.[14,153] Over the years, several mimetics have been produced, but none of them has all the anti-atherogenic functions of apoA-I. However, combining several mimetics can be a theoretical approach to mimic all anti-atherosclerotic properties of apoA-I.[154]

In vitro as well as animal studies have shown that several apoA-I mimetics improved HDL functionality (Table 10).[153] When cholesterol-enriched mouse macrophages were incubated in vitro with two mimetics (18A and 37pA), cholesterol efflux increased with 20%, which was comparable to the effects of adding lipid free apoA-I.[155] However, in following in vitro experiments the 37pA peptide was found to be cytotoxic with adverse effects on the integrity of the plasma membrane of HeLa cells.[156] All other mimetics reviewed here were not found to be toxic. Intraperitoneal injection of 20 μg 5F per day for 16 weeks in C57BL/6J mice reduced atherosclerotic lesion formation.[157] Also the peptide ATI-5261 reduced aortic lesion area and plaque lipid content, and increased cholesterol efflux in both LDL-R and apoE knockout mice.[158] In addition, in cells overexpressing ABCA1, ATI-5261 increased the specific ABCA1 dependent cholesterol efflux.[159] In rabbits ETC-642 had anti-inflammatory properties,[160] lowered oxidized LDL, shifted the HDL subfractions towards the pre-β fraction and increased cholesterol efflux in a human macrophage assay.[161]

Table 10 *Summary of apoA-I mimetics tested in cells, animals and humans.*

First author, Year	Mimetic	Duration	Model	Dose	Effect on
Yancey et al. (1995)[155]	18A, 37pA	1 till 24 hours	Mouse macrophages	1 mg/ml	- 20% ↓ in cholesterol efflux
Remaley et al. (2003)[156]	37pA	1 hour	ABCA1-transfected cells	0.4 mg/ml	- Cytotoxic
Garber et al. (2001)[157]	5F	16 weeks	Female C57BL/6J mice	20 μg/day	- 15.6 μm^2 ↑ atherosclerotic lesion formation
Bielicki et al. (2010)[158]	ATI-5261	6 weeks	Male LDL-R knockout mice	Daily intraperitoneal injections of 30 mg/kg	- 3.7% ↑ aortic lesion area - 8% ↑ plaque lipid content - ↓ cholesterol efflux
Hafiane et al. (2014)[159]	ATI-5261	2 hours	BHK cells overexpressing ABCA1	2 μg/ml	- ↓ the specific ABCA1 dependent lipid efflux - Mimics apoA-I metabolic pathway
Bielicki et al. (2010)[158]	ATI-5261	6 weeks	Male ApoE knockout mice	Every other day intraperitoneal injections of 30 mg/kg	- 45% ↑ in atherosclerosis - 20% ↑ plaque lipid content - 4 times ↓ RCT
Di Bartolo et al. (2011)[160]	ETC-642	6 weeks	Male New Zealand White rabbits	30 mg/kg	- ↓ anti-inflammatory properties
Iwata et al. (2011)[161]	ETC-642	12 weeks	Male and female Watanabe-heritable hyperlipidemic rabbits	50 mg/kg	- ↑ amount of oxidized LDL - Shift towards the pre-β HDL fraction
Tabet et al. (2010)[162]	5A	2, 12, 16 or 24 hours	Human coronary artery endothelial cells	1 mg/ml	- ↑ inflammation - ↑ oxidative stress
Tabet et al. (2010)[162]	5A	A single dose	Male New Zealand white rabbits	20 mg/kg	- ↑ inflammation - ↑ oxidative stress
Amar et al. (2010)[163]	5A	13 weeks	Female ApoE knockout mice	30 mg/kg	- 181% ↓ cholesterol efflux - 24% ↑ aortic plaque surface area
Chattopadhyay et al. (2013)[164]	6F	13 weeks	Female LDL-R knockout mice	45 mg/kg/day	- ↓ anti-inflammatory and antioxidant status - 4.1% to 1.9% ↑ aortic lesion area
Van Lenten et al. (2008)[165]	4F	1 hour	Lipid binding in human aortic endothelial cells	0.001-1 μg/ml	- ↓ cholesterol efflux capacity - ↓ anti-inflammatory properties - ↑ cellular LDL oxidation

Table 10 *Summary of apoA-I mimetics tested in cells, animals and humans. (continued)*

First author, Year	Mimetic	Duration	Model	Dose	Effect on
Navab et al. (2002)[14]	D-4F	6 weeks	Female LDL-R knockout mice	2.5 mg 2 times a day	- 79% ↑ atherosclerotic lesion development
Navab et al. (2002)[14]	D-4F	6 weeks	Female ApoE knockout mice	Added to the drinking water (>0.05 mg/L)	- 75% ↑ lesion development
Garber et al. (2001)[157]	D-4F	16 weeks	ApoE knockout mice	20 μg/day	- 75% ↑ atherosclerotic lesion development
Bloedon et al. (2008)[166]	D-4F	A single dose	Coronary artery diseased patients	30 vs. 100 vs. 300 vs. 500 mg	- ↓ anti-inflammatory activity of HDL

Both in cells and in New Zealand white rabbits, a single infusion of the 5A peptide reduced inflammation and oxidative stress.[162] Moreover, in apoE knockout mice an increased cholesterol efflux and a reduction of aortic plaque surface area was seen after 5A infusion.[163] Furthermore, the 6F peptide improved inflammatory biomarkers, antioxidant status and significantly reduced aortic lesion area in LDL-R knockout mice (Table 10).[164]

The most promising mimetic is 4F, which has not only been evaluated in vitro and in animal models, but also in a human clinical trial (Table 10). In vitro, 4F increased cholesterol efflux capacity and anti-inflammatory properties, as measured by a reduced monocyte chemotactic activity.[165] In human aortic endothelial cells, purified apoA-I and 4F both inhibited the cellular oxidation of LDL, which resulted in a lower pro-inflammatory activity. When mimetics are ingested, it is possible that enzymes in the gastrointestinal tract digest the proteins. Using D-amino acids (the use of the D-stereoisomer of amino acids for building mimetics instead of the L-stereoisomer, which is commonly found in nature) enables oral delivery due to resistance of the D-stereoisomers to human gastrointestinal proteolytic enzymes. In LDL-receptor knockout mice, which were fed a Western diet, D-4F reduced atherosclerotic lesion development with 79% and D-4F added to the drinking water reduced lesion development with 75%.[14] Intraperitoneal injection of 20 μg/day D-4F for 16 weeks in apoE knockout mice, fed an atherogenic diet, reduced atherosclerotic lesion development by 75%.[14,157] These studies show that oral D-4F, either provided via diet or water, is equally effective as compared to intraperitonel injections, which confirms the bioavailability and stability of the mimetic in the gastrointestinal tract and the circulation. When 50 patients with coronary artery disease received a single oral dose (30, 100, 300, 500 mg) of D-4F, the two highest doses increased the anti-inflammatory activity of the HDL fraction. However, no changes in lipids or lipoprotein concentrations were seen. D-4F was shown to be safe and well tolerated (Table 10).[166] Unfortunately, the effects of D-4F on cholesterol efflux in humans have not yet been investigated.

ApoA-I infusions

Besides apoA-I mimetics, apoA-I itself, either by using delipidated HDL or by using delipidated HDL combined with phospholipids, can be infused directly into the circulation. The theoretical advantage of using apoA-I or apoA-I-phospholipid complexes instead of using apoA-I mimetics is that the apoA-I protein is completely intact and still possesses all its biological functions and might therefore have a larger athero-protective effect. So far, three different forms of apoA-I have been tested i.e apoA-I Milano (MDCO-216), CSL-111 / CSL112 and CER-001 (Table 11).

ApoA-I Milano

Infusion of 40 mg recombinant apoA-I Milano (a mutant of human apoA-I) for 10 days in cholesterol-fed New Zealand White rabbits reduced intimal thickness, intima-to-media ratio, and the portion of intimal lesion covered by macrophages.[167] Infusion of 250, 500 or 1000 mg apoA-I Milano directly into the carotid artery of New Zealand White rabbits inhibited plaque progression and reduced the plaque area.[168] Eighteen injections every other day of 40 mg/kg apoA-I Milano in ApoE knockout mice showed a 40% decrease in lipid content and a 46% reduction in macrophage content in the lesion area.[169] In a randomized human controlled trial, 47 patients with acute coronary syndromes received for 5 weeks one infusion of placebo or recombinant apoA-I Milano/phospholipid complex (ETC-216) at 15 or 45 mg/kg per week. At the end of the study a significant reduction in atheroma volume was found in the high dose group.[170] This reduction in atheroma volume was accompanied by a reduction in external elastic membrane volume of the artery, but not with a change in lumen volume.[171] Recently, in a randomized controlled study, patients with stable coronary artery disease received 5 doses of 10, 20, 30, and 40 mg/kg MDCO-216 infusion. This resulted in a dose-dependent increase in apoA-I concentrations and a dose-dependent shift from small- to large-sized HDL particles.[172] Moreover, a profound increase in ABCA1-mediated cholesterol efflux was observed.[173] However, very recently the MILANO-PILOT study failed to slow down the regression of coronary atherosclerosis with 5 weekly infusions of 20 mg/kg MDCO-216 in 120 patients with acute coronary syndromes. In fact, significant reductions in HDL-C and apoA-I concentrations were observed and no effects on percent atheroma volume and total atheroma volume (Table 11).[174]

CSL-111/CSL112

In C57Bl/6 mice, a single injection with the reconstituted HDL particle CSL-111, i.e. a native human apoA-I/phosphatidylcholine complex, induced a dose- and time-dependent increase in human pre-β HDL particles and cholesterol efflux capacity.[175] Effects of CSL-111 have also been evaluated in several human studies. In one trial, 40 and 80 mg/kg CSL-111 was infused once a week for one month in 183 patients elected for coronary angiography. Treatment of the high dose group (80 mg/kg) was discontinued early, because some of the patients exceeded the upper level of alanine aminotransferase by 100-fold. The low dose group (40 mg/kg) showed a significant reduction in atheroma volume. However, this reduction was not significantly different from the decrease in the placebo group.[176] After this, the further development program of CSL-111 was discontinued because of the unfavorable hepatic abnormalities. As a follow-up, one phase I study has been performed using CSL112, which is a similar compound, but postulated without effects on liver function. In this study, a single

dose (5, 15, 40, 70, 105 or 135 mg/kg) or multiple doses for 4 weeks (3.4 or 6.7 g once a week or 3.4 g twice a week) of CSL112 was administrated intravenously to healthy volunteers. Both the single and multiple doses, dose dependently increased serum apoA-I concentrations above baseline for 3 days or longer and increased serum HDL-C concentrations. Moreover, also pre-β HDL particle concentrations and cholesterol efflux capacity were increased. In the single dose study, dose-dependent effects were found on HDL-C.[177,178] Recently, two studies showed that CSL112 was indeed safe for human consumption, with no effects on liver function parameters.[179,180] In the first study, patients with atherosclerosis were given infusions of 1.7, 3.4, 6.8 g CSL112 or placebo. The CSL112 infusions resulted in a dose-dependent increase in apoA-I and total cholesterol efflux.[180] In the second study patients with myocardial infarction received infusions of 2 or 6 g CSL112 or placebo for 4 weeks. Here also a dose dependent increased in HDL-C, apoA-I and cholesterol efflux was shown (Table 11).[179]

CER-001

Mice infused with 10 mg/kg CER-001 showed an increased RCT and cholesterol efflux. In LDL-R knockout mice fed a high-cholesterol diet, infusion of 5 and 10 doses of CER-001 given every 2 days reduced lesion size and lesion lipid content.[181] Besides animal studies, effects of CER-001 have already been evaluated in a number of human clinical trials. In one study, 417 patients with acute coronary syndromes were randomized for 6 weekly infusions of 3, 6, 12 mg/kg CER-001 or placebo. No changes in atheroma volumes were found. It was speculated that a higher dose or a different patient group would have shown more positive results.[182] In a recent human study, 9 infusions of 8 mg/kg CER-001 were given twice weekly for 28 days to 7 patients with familial hypoalphalipoproteinemia, who were severely deficient in HDL. In this patient group, CER-001 significantly increased serum apoA-I and HDL-C concentrations and reduced atherosclerotic lesion size, measured using Magnetic Resonance Imaging. Moreover, an increase in cholesterol efflux from macrophages and a higher fecal neutral sterol excretion was seen, which may indicate improved RCT.[183] Additionally, 12 biweekly infusions with 8 mg/kg CER-001 showed increased apoA-I concentrations, a decrease in vessel wall area and a trend towards a reduction in vessel wall thickness.[184] Recently, a study evaluated the effects of 3 mg/kg CER-001, in patients with atherosclerotic carotid artery disease, and showed increased apoA-I concentrations, with a simultaneously increased cholesterol efflux capacity (Table 11).[185] Unfortunately, preliminary data of a recent clinical trial in patients with coronary atherosclerosis did not show beneficial effects of CER-001 on atheroma volume and LDL-C.[186]

Table 11 *Summery of all studies testing apoA-I infusions in cells, animals and human*

First author, Year	Infusion	Duration	Model	Dose	Effect on
Ameli et al. (1994)[167]	ApoA-I Milano	10 days	Male new Zealand White rabbits	40 mg	- 0.49 and 1.2 mm^2 ↑ intimal thickness - 0.8 and 1.4 ↑ intima-to-media ratio - 34.1% ↑ macrophage content
Chiesa et al. (2002)[168]	ApoA-I Milano	A single dose	Male new Zealand White rabbits	250 vs. 500 vs. 1000 mg	- 0, 29 and 13% ↑ plaque progression - 30% ↑ plaque area by highest dose
Shah et al. (1998)[169]	ApoA-I Milano	18 infusions every other day	ApoE knockout mice	40 mg/kg	- 40% ↑ in lipid content - 46% ↑ in macrophage content
Nissen et al (2003)[170]	ApoA-I Milano	One infusion for 5 weeks	Patients with acute coronary syndromes	15 vs. 45 mg/kg	- 15.1 mm^3 and 12.6 mm^3 ↑ in atheroma volume
Kempen et al. (2016) and Kallend et al. (2016) [172,173]	ApoA-I Milano	5 doses during 2 hours	Patients with stable coronary artery disease	10 vs. 20 vs. 30 vs. 40 mg/kg	- Dose-dependent ↓ in apoA-I concentrations - Dose-dependent shift from small- to large-sized HDL particles. - ↓ in ABCA1-mediated cholesterol efflux
Nicholls et al. (2016)[174]	ApoA-I Milano	5 weekly doses	120 patients with a recent acute coronary syndrome	20 mg/kg	- 7.8 and 5.3% ↑ in fasting HDL-C and apoA-I concentrations - No effects on percent and total atheroma volume
Chen et al. (2012)[175]	CSL-111	A single dose	Male C57Bl/6 mice	50 vs. 100 vs. 200 mg/kg	- Dose- and time-dependent ↓ in pre-β HDL particles - Dose- and time-dependent ↓ in cholesterol efflux
Tardy et al. (2007)[181]	CSL-111	Once a week for a month	Patients elected for coronary angiography	40 vs. 80 mg/kg	- Abnormalities in liver function test - 3.4% ↑ in atheroma volume
Easton et al. (2014) and Gille et al. (2014) [177,178]	CSL112	A single dose	Healthy volunteers	5 vs. 15 vs. 40 vs. 70 vs. 105 vs. 135 mg/kg	- ↓ in apoA-I concentrations for 3 days or longer - 81% ↓ in HDL concentrations - ↓ pre-β HDL particle concentrations - 2.9 fold ↓ in cholesterol efflux

Table 11 *Summery of all studies testing apoA-I infusions in cells, animals and human (continued)*

First author, Year	Infusion	Duration	Model	Dose	Effect on
Easton et al. (2014) and Gille et al. (2014)[177,178]	CSL112	Once or twice weekly for 4 weeks	Healthy volunteers	3.4 vs.6.7 g once a week vs.3.4 g twice a week	- ↓ in apoA-I concentrations for 3 days or longer - ↓ pre-β HDL particle concentrations - 2.6 fold ↓ in cholesterol efflux
Tricoci et al. (2015)[180]	CSL112	A single dose	Patients with atherosclerosis	1.7 vs. 3.4 vs. 6.8 g	- No elevations in alanine aminotransferase or aspartate aminotransferase - No serious adverse events. - Dose-dependent ↓ in apoA-I concentrations and total cholesterol efflux
Gibson et al. (2016)[179]	CSL112	4 weekly infusions	Patients with myocardial infarction	0 vs. 2 vs. 6 g	- Safe for use - Dose-dependent ↓ in fasting apoA-I, HDL-C concentrations and cholesterol efflux
Tardy et al. (2014)[181]	CER-001	Infusion of 5 and 10 doses every 2 days	Male LDL-R knockout mice	10 mg/kg	- 17% and 32% ↑ in lesion size - 17% and 23% ↑ in lesion lipid content
Tardif et al. (2014)[182]	CER-001	6 weekly infusions	Patients with acute coronary syndromes	3 vs. 6 vs. 12 mg/kg	- No changes in atheroma volumes
Kootte et al. (2015)[183]	CER-001	9 infusions twice weekly for 28 days	Patients with Familial Hypoalpha-lipoproteinemia	8 mg/kg	- 94% ↓ in apoA-I concentrations - 117% ↓ in HDL-C concentrations - 8.8% ↑ atherosclerotic lesion size - 44% ↓ in cholesterol efflux - ↓ fecal neutral sterol excretion
Hovingh et al. (2015)[184]	CER-001	12 infusions twice weekly	Patients with homozygous familial hypercholesterolemia	8 mg/kg	- 13% ↓ in apoA-I concentrations * - 2.8% ↑ in vessel wall area * - Trend toward ↑ in vessel wall thickness
Zheng et al. (2016)[185]	CER-001	A single dose	Patients with atherosclerotic carotid artery disease	3 mg/kg	- 8.7% ↓ in apoA-I concentrations * - 13.8% ↓ in the cholesterol efflux capacity
Nicholls et al. 2017[186]	CER-001	10 weekly infusions	Coronary artery diseased patients		- No difference in atheroma volume - No difference in LDL-C

Table 11 Summery of all studies testing apoA-I infusions in cells, animals and human (continued)

First author, Year	Infusion	Duration	Model	Dose	Effect on
Navab et al. (2002)[14]	D-4F	6 weeks	Female LDL-R knockout mice	2.5 mg 2 times a day	- 79% ↑ atherosclerotic lesion development
Navab et al. (2002) [14]	D-4F	6 weeks	Female ApoE knockout mice	Added to the drinking water (>0.05 mg/L)	- 75% ↑ lesion development
Garber et al. (2001)[157]	D-4F	16 weeks	ApoE knockout mice	20μg/day	- 75% ↑ atherosclerotic lesion development
Bloedon et al. (2008)[166]	D-4F	A single dose	Coronary artery diseased patients	30 vs. 100 vs. 300 vs. 500 mg	- ↓ anti-inflammatory activity of HDL

Others

RVX-208

The first class of compounds affecting apoA-I metabolism refers to the apoA-I transcriptional upregulator RVX-208. RVX-208 is an oral, small synthetic quinazoline molecule, which binds bromo and extra terminal (BET) proteins and upregulates apoA-I gene transcription via an epigenetic mechanism.

In vitro, incubating HEPG2 cells with RVX-208 increased apoA-I mRNA expression, and intracellular and extracellular apoA-I protein mass. Treating three African Green Monkeys with 60 mg/kg RVX-208 orally daily for 63 days, increased serum apoA-I and HDL-C concentrations with 60% and 97% respectively. Moreover, also HDL profiles were changed; pre-β1-LpA-I and larger α1-LpA-I subpopulations were significantly increased, whereas α2-LpA-I was significantly decreased. In the treated animals also cholesterol efflux, measured in vitro using J774 cells, was significantly increased.[187] Furthermore, in hyperlipidemic apoE knockout mice, 150 mg/kg RVX-208 for 12 weeks significantly reduced aortic lesion formation, which was accompanied by an increase in serum HDL-C concentrations and a decrease in LDL-C concentrations, adhesion molecules and cytokines. However, no significant changes in apoA-I concentrations were observed.[188]

In the first human clinical trial, 18 healthy subjects received varying and multiple doses (1 to 20 mg/kg per day) of RVX-208 or placebo for 7 days. Plasma apoA-I concentrations were increased, and more importantly, an increase in pre-β1-HDL concentrations and a higher ABCA1-mediated cholesterol efflux was demonstrated.[187] The outcome of the recent phase 2 randomised placebo controlled clinical ASSERT trial, evaluating the effect of RVX-208 on serum apoA-I concentrations and CHD risk in human, was less positive. In that study, 299 patients with stable coronary artery disease received placebo or RVX-208 at three different dosages (50, 100, 150 mg) twice daily for 12 weeks. Only a non-significant increase in serum apoA-I concentrations was found. Unfortunately, HDL functionality and cholesterol efflux capacity were not studied.[189] A second study using RVX-208 is the phase 2b clinical trial SUSTAIN. In this trial, 172 statin-treated patients (Rosuvastatin or Atorvastatin) with low serum HDL-C concentrations were treated with 200 mg/day RVX-208 for 24 weeks. Both serum apoA-I concentrations as well as HDL particle numbers increased significantly. Furthermore, RVX-208 was found to be safe for oral use.[190] In another phase 2 clinical trial, the ASSURE study, 323 statin (Rosuvastatin or Atorstatin) treated patients with coronary artery disease and low serum HDL-C concentrations received 100 mg RVX-208 twice daily for 26 weeks. However, no significant reductions in atheroma volume or increases in HDL-C and apoA-I concentrations were seen.[31] Finally, a recent study in subjects with pre-diabetes showed that 100 mg RVX-208 for 29-33 days did not

increase HDL-C and apoA-I concentrations, while it increased the concentration of medium-sized HDL and decreased the concentration of small-sized HDL particles. Furthermore, RVX-208 delayed and reduced oral glucose absorption and endogenous glucose production (Table 12).[191]

LCAT infusion

The first human study investigating the effects of lecithin:cholesterol acyltransferase (LCAT) infusion investigated only one patient with familial LCAT deficiency. Recombinant human LCAT was infused 3 times for 1 hour in a dose optimization phase (0.3, 3.0, and 9.0 mg/kg) and after this 1 to 2 weekly infusions were given of 3.0 or 9.0 mg/kg for 7 months. LCAT infusion improved renal function, increased apoA-I, HDL-C and in a lesser extend LDL-C. Furthermore, after infusion, postprandial triacylglycerol concentrations decreased.[192] These results are promising; however, before drawing conclusions about LCAT infusion clinical trials including more patients should be done.

Conclusion

Alcohol consumption increases fasting apoA-I concentrations and may improve cholesterol efflux, possibly via increasing apoA-I PR and decreasing FCR. Further, replacement of carbohydrates for SFA, cis-MUFA, cis-PUFA and TFA increases fasting apoA-I concentrations. The effects of the various SFA are different, since lauric, palmitic and myristic acids increase apoA-I concentrations, while stearic acid does not. The different fatty acids affect apoA-I metabolism differently, but results are conflicting. Therefore more studies are needed to better understand the effects of the various macronutrients on apoA-I kinetics.

Coffee, caffeine, tea, omega 3 fatty acid, fish, nuts, plant sterol and stanol, different soy proteins and isoflavones isolated from soy do not change fasting apoA-I concentrations. Moreover, the effects of the various types of fibers may be different; the consumption of diets rich in wheat germ did not modify apoA-I concentrations, while the consumption of diets rich in psyllium, arabinoxylan and flaxseed may decrease fasting apoA-I concentrations. However, these types of fibers have only been examined in a limited number of studies. Therefore, we conclude that fiber consumption does not have a profound impact on fasting apoA-I concentrations.

Finally, five other food components showed a promising increase in fasting apoA-I concentrations; citrus, vitamin D, theobromine, orange juice, and a high dose of grape pomace and omija fruit. However, these findings need to be confirmed in future

Table 12 *Summery of all studies testing RVX-208 and lecithin:cholesterol acyltransferase (LCAT) infusions in cells, animals and human*

First author, year	Infusion	Duration	Model	Dose	Effects on
Bailey et al. (2010)[187]	RVX-208		HEBG2 cells	0 to 60 μmol/l	- ↓ apoA-I mRNA and intra and extracellular protein mass
Bailey et al (2010)[186]	RVX-208	63 days	African Green Monkeys	60 mg/kg	- 60% ↓ in apoA-I concentrations - 97% ↓ in HDL-C concentrations - Changed HDL profile - ↓ in cholesterol efflux
Jahagirdar et al. (2014)[188]	RVX-208	12 weeks	Hyperlipidemic apoE knockout mice	150 mg/kg	- ↑ aortic lesion formation - 2 fold ↓ HDL-C concentrations - 50% ↑ in LDL-C concentrations - No change in apoA-I concentrations - ↑inflammation
Bailey et al. (2010) [188]	RVX-208	7 days	Healthy subjects	1 to 20 mg/kg/day	- 11% ↓ in apoA-I concentrations - 11% ↓ in HDL-C concentrations - 42% ↓ in pre-β1-HDL concentrations - 11% ↓ in ABCA1-mediated cholesterol efflux
Nicholls et al. (2011)[189]	RVX-208	Twice daily for 12 weeks	Patients with stable coronary artery disease	50 vs. 100 vs. 150 mg	- No difference in apoA-I concentrations
Gilham et al. (2016)[189]	RVX-208	24 weeks	Statin-treated patients with low HDL-C concentrations	200 mg/day	- ↓ in apoA-I concentrations - ↓ HDL particle number - Safe for oral use
Nicholls et al. (2016)[31]	RVX-208	26 weeks	Statin-treated patients with coronary artery disease and low HDL-C concentrations	100 mg twice daily	- No difference in atheroma volume, HDL-C and apoA-I concentrations

Table 12 Summery of all studies testing RVX-208 and lecithin:cholesterol acyltransferase (LCAT) infusions in cells, animals and human (continued)

First author, year	Infusion	Duration	Model	Dose	Effects on
Siebel et al. (2016)[191]	RVX-208	29-33 days	20 males with prediabetes	100 mg	- No change in HDL-C and apoA-I concentrations - 11% ↓ in medium size HDL particles - 10% ↑ in small size HDL particles - Later and ↓ glucose peak - ↑ endogenous glucose production
Shamburek et al. 2016[192]	Recombinant human LCAT	7 months	1 patient with LCAT deficiency	Optimization phase: 3 times 1 hour 0.3, 3.0, and 9.0 mg/kg. Maintenance phase: every 1 to 2 weeks 3.0 or 9.0 mg/kg	- ↑ apoA-I, HDL-C and to a lesser extent LDL-C - ↓ postprandial triacylglycerol concentrations

studies. Additional research is also needed to examine the effects of these products or food components not only on apoA-I kinetics, but also on HDL functionality.

Overall, all three categories of pharmacological approaches showed that targeting apoA-I concentrations and/or HDL functionality by a pharmacologic approach can increase apoA-I functionality and might improve CHD risk markers, including vessel wall characteristics and inflammation. The mimetic D-4F is promising, but clinical studies are required to investigate the effects on HDL functionality. The CSL112 and CER-001 are the most promising of the infusion therapies, but studies are needed to investigate the effects of CSL112 on CHD risk markers, including vessel wall characteristics and inflammation. Unfortunately, recent clinical studies showed no improvement in CHD risk markers after apoA-I Milano or RVX-208 therapy.

Although we cannot exclude that we have missed studies during the systematic searches and studies with positive results are overrepresented, we conclude that both dietary components and pharmacological approaches can be used to increase apoA-I concentrations. For the dietary components in particular, more knowledge about underlying mechanisms is necessary, as increasing apoA-I per se does not necessarily translate into a reduced CHD risk.

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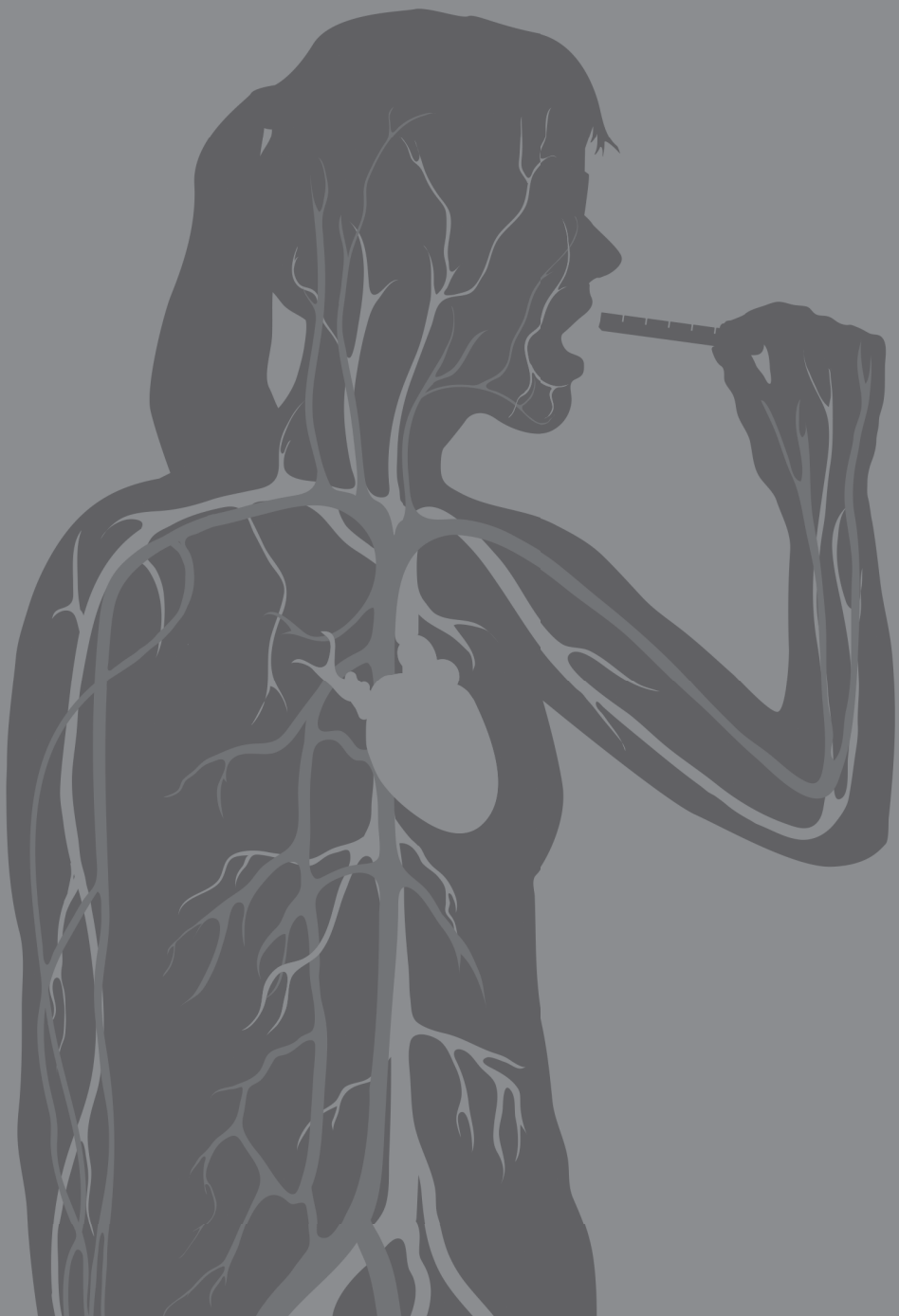

Chapter 3

An acute intake of theobromine does not change postprandial lipid metabolism, whereas a high-fat meal lowers chylomicron particle number

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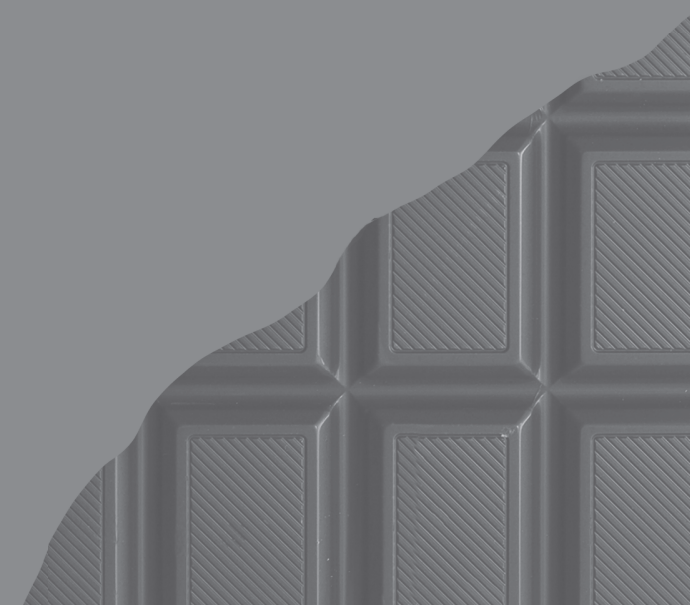

Abstract

Postprandial responses predict cardiovascular disease risk. However, only a few studies have compared acute postprandial effects of a low-fat, high-carbohydrate (LF) meal with a high-fat, low-carbohydrate (HF) meal. Furthermore, theobromine has favorably affected fasting lipids, but postprandial effects are unknown. As both fat and theobromine have been reported to increase fasting apolipoprotein A-I (apoA-I) concentrations, the main hypothesis of this randomized, double blind crossover study was that acute consumption of a HF meal and a theobromine meal increased postprandial apoA-I concentrations, when compared with a LF meal. Theobromine was added to the LF meal. Nine healthy men completed the study. After meal intake, blood was sampled frequently for 4 hours. Postprandial apoA-I concentrations were comparable after intake of the three meals. Apolipoprotein B48 (apoB48) curves, however, were significantly lower and those of triacylglycerol (TAG) significantly higher after HF as compared with LF consumption. Postprandial free fatty acid concentrations decreased less, and glucose and insulin concentrations increased less after HF meal consumption. Except for an increase in the incremental area under the curve (iAUC) for insulin, theobromine did not modify responses of the LF meal. These data shows that acute HF and theobromine consumption does not change postprandial apoA-I concentrations. Furthermore, acute HF consumption had divergent effects on postprandial apoB48 and TAG responses, suggesting the formation of less, but larger chylomicrons after HF intake. Finally, except for an increase in the iAUC for insulin, acute theobromine consumption did not modify the postprandial responses of the LF meal.

Introduction

Increasing evidence suggests that not only fasting lipid and glucose concentrations, but also a disturbed postprandial triacylglycerol (TAG) and glucose metabolism are important risk markers for cardiovascular disease (CVD).[1] In fact, Bansal and colleagues. have shown that the number of CVD events was more strongly related to postprandial TAG concentrations (2-4 hours after meal consumption) than to fasting TAG concentrations.[2] Not only postprandial TAG, but also overall glycemic control[3] and postprandial glycaemia[4] are related to CVD risk. Moreover, a tight glycemic control of diabetic patients clearly lowered the risk for CVD events.[5]

Although a substantial number of studies have examined the effects of changing the amount and types of fat in the diet on fasting lipid, lipoprotein and glucose concentrations,[6,7] only a few studies have addressed their effects during the postprandial phase. It is known that fat intake dose-dependently increases postprandial plasma TAG concentrations,[8,9] especially in the chylomicron fraction.[8] In addition, a smaller decrease in postprandial free fatty acid (FFA) concentrations was observed after consuming a high-fat meal as compared with a fat-free meal.[10]

Besides changing the amount and type of fat in the diet, it is also possible to enrich the diet with functional ingredients to improve metabolic profiles related to CVD risk. Several studies have shown that consuming cocoa or cocoa-containing products improves fasting lipid profiles.[11] Theobromine is one of the compounds in cocoa, which may contribute to these beneficial effects. In fact, Neufingerl et al. have shown that a higher intake of theobromine improved dyslipidemia by increasing fasting serum high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (apoA-I) concentrations and by lowering fasting serum apolipoprotein B (apoB) and low-density lipoprotein cholesterol (LDL-C) concentrations.[12]

The effects of theobromine on postprandial lipid, apolipoprotein and glucose metabolism have not been explored yet. We now hypothesized that both the HF meal and theobromine consumption increased postprandial apoA-I concentrations when compared with a LF meal. Therefore, we have evaluated side-by-side the acute effects of a low-fat/high-carbohydrate (LF), a high-fat/low-carbohydrate (HF) meal as well as adding theobromine to a LF meal (LF-TB) on postprandial lipid, apolipoprotein and glucose metabolism in apparently healthy men. The theobromine was added to the LF meal to minimize the possibility that dietary fat content masked the potential theobromine effect on apoA-I.

Material and Methods

Study population

Ten apparently healthy men from Maastricht and surrounding areas were recruited via posters in the university and hospital buildings. They were invited for a screening visit if they met the following inclusion criteria: 18-60 years of age, BMI between 20 and 30 kg/m^2, stable body weight (weight gain or loss < 3 kg in the previous 3 months), no use of lipid-lowering medication or a prescribed diet, no abuse of alcohol or drugs, no smoking, no diabetes, no history of coronary artery disease, no history of gastrointestinal disorders and no participation in another lifestyle or pharmaceutical intervention study for the past 30 days. During the screening visit, body weight, height and blood pressure were determined. Blood pressure was measured in fourfold using an Omron M7 (Omron Healthycare Europe B.V., Hoofddorp, the Netherlands). The first blood pressure measurement was discarded and the last three measurements were averaged. During screening, fasting blood was sampled for analysis of serum total cholesterol and plasma glucose concentrations. Subjects were excluded if fasting serum total cholesterol concentrations were > 8.0 mmol/L or plasma glucose concentrations were > 7.0 mmol/L. Once included, subjects were asked not to change their dietary habits, level of physical exercise and alcohol intake for the entire duration of the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. The study protocol was approved by the Medical Ethical Committee of the Maastricht University Centre$^+$. All participants gave their written informed consent before entering the study. Finally, the study was registered on clinicaltrials.gov under study number NCT02085109.

Study design

This randomized double-blind crossover trial consisted of three test days, each separated by a one week wash-out period. Two weeks before the start of the study, subjects were instructed to avoid products containing cocoa till the end of the study period. For this, they received a detailed list with food products from our research dietician. In addition, two weeks before the start of the study, also the consumption of caffeine containing drinks was restricted, i.e. to a maximum of 4 cups a day, since theobromine is a metabolite of caffeine. Moreover, participants were instructed to keep their caffeine intake constant during the study. In theory, these 4 caffeine-containing drinks could have resulted in the formation of maximally 80 mg of theobromine,[13] which is ± 9.4% of the additional experimental theobromine intake during the study. Finally, during the test days caffeine intake was completely prohibited. To minimize differences in dietary background between the three test days, we provided all subjects with a standard low-fat dinner the evening before

each test day, which consisted of a commercially available macaroni dish, 3 crackers, and a dairy drink. In addition, they were asked not to change their dietary habits or levels of physical activity. Furthermore, participants recorded in diaries any signs of illness, medication used, alcohol consumption, any deviations of the study protocol and other complaints. They also recorded their food intake to estimate their habitual energy and nutrient intakes during the week before every test day by completing a food frequency questionnaire (FFQ). These FFQs were checked and processed by our research dietician using the Dutch Nutrient databank[14] to check whether dietary energy and nutrient intakes had remained stable during the study.

Test days

Subjects came to the University in the morning in fasting condition, which meant that after the intake of the standardized dinner on the preceding evening, they had not consumed any foods or drinks, except for water, till the morning of the test day. Participants arrived at our department by public transport or by car to reduce physical activity as much as possible. Upon arrival, after 15 min of rest, an intravenous cannula was inserted into the anticubital vein and a fasting blood sample was collected (T0). Next, subjects were asked to consume a shake within 10 minutes. Each of the three test days the participants had to consume a different shake in randomized order; one high-fat/low-carbohydrates (HF) shake, one low-fat/high-carbohydrates (LF) shake and one low-fat/high-carbohydrates shake enriched with 850 mg theobromine (LF-TB) (Table 1). The theobromine powder (850 mg; Fagron, Uitgeest, the Netherlands) was added as the last ingredient to the blender jar before it was thoroughly mixed with the LF shake. The volume of the LF shake was 613 ml and of the HF shake was 453 ml. To standardize the total volume, a glass of 160 ml of water was given together with the HF shake. Protein levels of the three shakes were comparable, which means that the only difference between the HF and LF shakes were the levels of fat and carbohydrates (Table 1). As the shakes were prepared by the research dietician, both the researchers who performed the measurements and the participants were blinded for the interventions.

After consuming the shakes, the volunteers were not allowed to eat or drink anything except water for the next 5 hours. Postprandial blood samples were taken at T = 15 (T15), T = 30 (T30), T = 45 (T45), T = 60 (T60), T = 90 (T90), T = 120 (T120), T = 180 (T180) and T = 240 (T240) minutes after shake consumption.

Blood sampling

Blood was sampled in serum, EDTA- and NaF-containing vacutainer tubes (Becton Dickinson, Breda, The Netherlands). The EDTA and NaF tubes were placed on ice directly after sampling and were centrifuged at 1300 x g for 15 min at 4˚C within 60

min after sampling to obtain EDTA and NaF plasma. Serum tubes were allowed to clot for 1 hour at 20˚C, followed by centrifugation at 1300 x g for 15 min at 20˚C to obtain serum. Serum and plasma aliquots were stored at -80˚C until analyses.

Table 1 *Nutrient composition of the LF, HF and LF-TB shakes*[1]

Nutrient	HF	LF	LF-TB
Energy (kcal)	965	956	956
Protein (g)	17.9	19.4	19.4
(E%)[1]	7	8	8
Carbohydrates (g)	85.7	193.7	193.7
(E%)	35	81	81
Mono- and disaccharides (g)	45.6	144.9	144.9
Polysaccharides (g)	40.1	48.8	48.8
Total fat (g)	60.6	10.5	10.5
(E%)	56	10	10
Saturated fatty acids (g)	36.0	3.2	3.2
Monounsaturated fatty acids (g)	18.7	4.0	4.0
Polyunsaturated fatty acids (g)	4.1	1.1	1.1
Cholesterol (mg)	341	334	334
Theobromine (mg)	0	0	850

[1] *Abbreviations: LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine, E%: energy percent.*

Analyses

All samples from one subject were analyzed within the same analytical run at the end of the study. Plasma glucose (Roche Diagnostic Systems, Woerden, the Netherlands) and FFA (Wako Biochemicals, Richmond, USA) concentrations were measured in NaF plasma at all indicated time points during the postprandial tests. At the same time points, serum insulin concentrations were determined with a human insulin-specific radioimmunoassay (RIA) kit (Linco Research, Missouri, USA). Serum TAG concentrations (GPO Trinder; Sigma-Aldrich, Missouri, USA) with correction for free glycerol were measured at T0, T30, T60, T90, T120, T180 and T240. At these time points, also serum apolipoprotein B48 (apoB48) concentrations were measured with an ELISA kit (Shibayagi, Gunma, Japan). ApoA-1 and apolipoprotein B100 (apoB100) were analyzed using highly sensitive immunoturbidimetric assays (Horiba ABX, Montpellier Cedex 4 France) in serum at all time points during postprandial tests.

Statistical analyses

Power calculations were based on an expected true difference in postprandial apoA-I concentrations between diets at a certain time point of 0.07 g/L. This value was based on the observations of Neufingerl et al. who found this difference in fasting

apoA-I concentrations after 4-weeks consuming 850 mg of theobromine/day.[12] When a within-subject variability of 0.06 g/L was used, the statistical power to detect this difference between the diets was 80% (α=0.017), when 8 subjects successfully completed the study. As the expected drop-out rate was 20%, we recruited 10 men.

All data is presented as means ± SE unless otherwise indicated. Differences in fasting concentrations and FFQ results were evaluated by repeated-measures analysis of variance (ANOVA) with diet as within subject factor and Bonferroni as post hoc test.

To test differences in postprandial responses after meal intakes, two different analyses were performed. First, the incremental areas under the curves (iAUC) were calculated using the trapezoidal rule,[15] for the overall postprandial TAG, apoB48, glucose and insulin responses. Because the oral glucose tolerance test (OGTT) is normally performed till T120,[16,17] the iAUCs for glucose and insulin were calculated for T0-T120 as well as for T0-T240. For FFA, the decremental area under the curve (dAUC) was calculated. iAUC and dAUC values were not normally distributed, as tested with the Shapiro-Wilk test, and are reported as medians with ranges. Differences between meal effects were tested for statistical significance using a Friedman test. When the Friedman test was significant, a Wilcoxon signed rank test was performed to compare the diets pairwise. Second, changes from baseline were analyzed by linear mixed models with diet and time as fixed factors and a diet x time interaction. If this interaction was not significant, it was omitted from the model. If diet was significant, the diets were compared pairwise. If time was significant, time points were compared to baseline values. If the interaction term was significant, differences between the diets were tested at each individual time point. Bonferroni's corrections for multiple comparisons were used.

Results were considered to be statistically significant if $p \leq 0.05$. All statistical analyses were performed using SPSS 20.0 for Mac (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

All ten men that started the study completed the three postprandial test days. However, one subject was excluded from the statistical analysis due to protocol violation, since he was non-fasting at one of the test days, as indicated by clearly increased fasting TAG, glucose and apoB48 concentrations (Figure 1). Baseline characteristics of the remaining nine men are shown in Table 2.

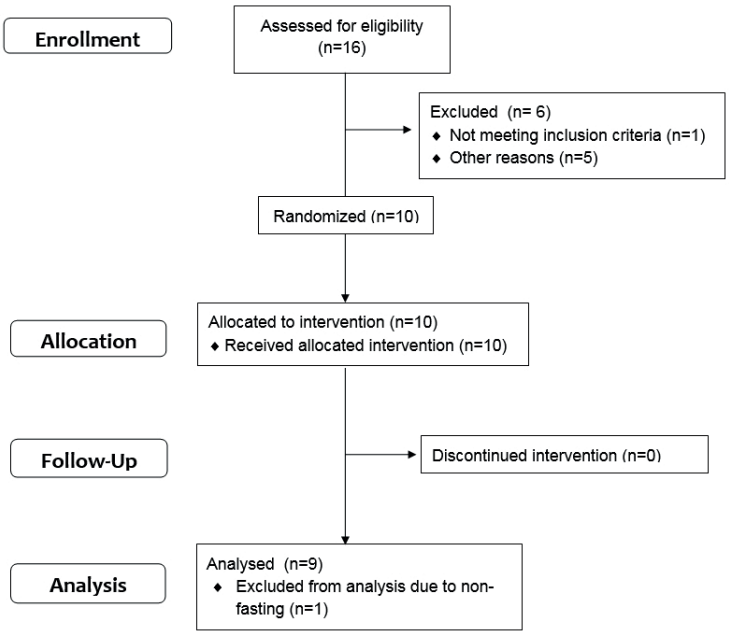

Figure 1 *Flow chart of participant inclusion throughout the study*

FFQ

Based on the FFQ data, there were no differences in energy, fat, carbohydrate, protein, cholesterol or fiber intakes during the weeks preceding each of the three test days (Table 3).

Table 2 *Baseline characteristics of the participants*[1]

	Mean±SD
Age (years)	36 ± 15
BMI (kg/m^2) [1]	24.4 ± 1.9
Serum total cholesterol (mmol/L)	5.1 ± 0.9
Plasma glucose (mmol/L)	5.0 ± 0.3
Systolic blood pressure (mmHg)	129 ± 17
Diastolic blood pressure (mmHg)	76 ± 15

[1] *Data are reported as means ± SD, n=9.*
Abbreviations: BMI: Body mass index

Table 3 *Composition of the habitual diets as consumed in the week before the LF, HF and LF-TB test days*[1]

	HF	LF	LF-TB
Energy (kcal/day)	2643 ± 534	2699 ± 742	2865 ± 542
Protein (E%)	15.4 ± 2.7	15.5 ± 3.5	15.2 ± 1.7
Carbohydrates (E%)	39.5 ± 5.8	39.3 ± 5.5	40.8 ± 6.3
Total fat (E%)	38.1 ± 6.0	39.8 ± 8.0	38.0 ± 7.2
Saturated fatty acids (E%)	11.5 ± 2.5	12.6 ± 3.5	11.9 ± 2.8
Monounsaturated fatty acids (E%)	14.8 ± 1.9	14.8 ± 4.4	14.4 ± 2.7
Polyunsaturated fatty acids (E%)	8.3 ± 2.2	8.8 ± 2.2	8.5 ± 1.9
Alcohol (E%)	5.0 ± 3.2	3.5 ± 2.8	3.8 ± 2.0
Cholesterol (mg/day)	254 ± 110	275 ± 149	272 ± 107
Fiber (g/day)	23.5 ± 7.8	26.9 ± 8.7	27.3 ± 4.7

[1] *Data are reported as means ± SD, n=9.*

ANOVA with diet as within subject factor and Bonferroni as post hoc test were conducted to determine significance between the diets.

Abbreviations: LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine.

Postprandial lipids, apolipoproteins, glucose and insulin

Fasting serum lipid, apolipoprotein, glucose and insulin concentrations at the start of the three test days were comparable (Table 4).

For both apoA-I and apoB100, no significant diet effects were observed. However, changes in apoA-I and apoB100 showed a significant time effect (Figure 2). After meal consumption, serum apoA-I concentrations were lowered at T120 from

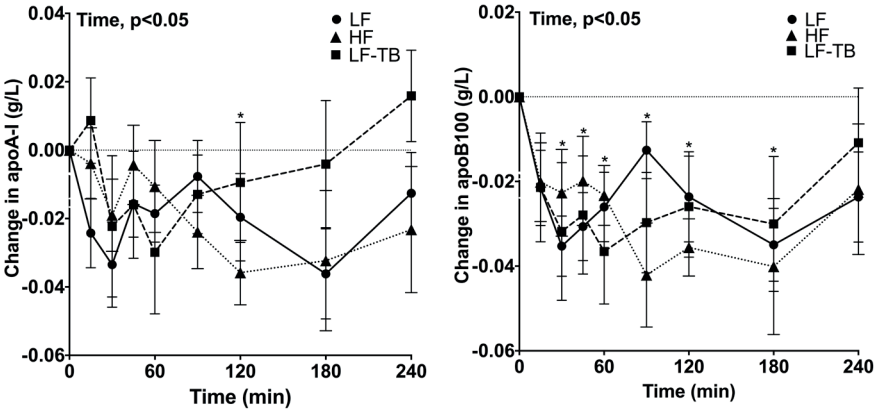

Figure 2 *A. Mean changes in serum apoA-I concentrations after LF, HF or LF-TB consumption. B. Mean changes in serum apoB100 concentrations after LF, HF or LF-TB consumption.*

Data are reported as mean changes ± SE, n=9.

*Linear mixed model procedures were conducted to determine significance between the diets, significantly different from the HF diet * (p<0.05).*

Abbreviations: apoA-I: apolipoprotein A-I, LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine, apoB100: apolipoprotein B100.

baseline ($p = 0.028$). Postprandial apoB100 concentrations were lowered between T30-T180 from baseline ($p \leq 0.044$ at all time points).

Table 4 *Fasting TAG, FFA, apoB48, apoA-I, apoB100, glucose and insulin concentrations at the start of the LF, HF and LF-TB test days*[1]

	HF	LF	LF-TB
TAG (mmol/l)	1.03 ± 0.44	1.09 ± 0.29	1.06 ± 0.37
ApoB48 (ng/ml)	6090 ± 2790	6879 ± 2336	6524 ± 2346
FFA (μmol/l)	484 ± 197	457 ± 175	490 ± 133
ApoA-I (g/l)	1.39 ± 0.89	1.39 ± 0.21	1.39 ± 0.26
ApoB100 (g/l)	0.96 ± 0.18	1.01 ± 0.16	0.96 ± 0.17
Glucose (mmol/l)	5.62 ± 0.44	5.50 ± 0.32	5.72 ± 0.30
Insulin (μU/ml)	11.7 ± 4.0	11.1 ± 2.6	10.2 ± 2.7

[1] *Data are reported as means ± SD, n=9.*
ANOVA with diet as within subject factor and Bonferroni as post hoc test were conducted to determine significance between the test days.
Abbreviations: TAG: triacylglycerol, FFA: free fatty acids, apoB48: apolipoprotein B48, apoA-I: apolipoprotein A-I, apoB100: apolipoprotein B100 LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine.

For changes in postprandial TAG concentrations, the diet x time interaction reached statistical significance (Figure 3). Increases in serum TAG concentrations were higher after the HF meal as compared with the LF and LF-TB meals at T120 ($p = 0.002$ and $p < 0.001$, respectively), T180 (both $p<0.001$) and T240 ($p = 0.004$ and $p = 0.012$, respectively). As shown in Table 5, the iAUC for serum TAG concentrations was higher after the HF as compared with the LF and LF-TB meals ($p = 0.038$ and $p = 0.011$ respectively).

As shown in Figure 3, the diet x time interaction was also significant for changes in serum apoB48 concentrations ($p = 0.042$). Changes in apoB48 concentrations were less pronounced after the HF meal as compared with the LF and LF-TB meals at T180 (both $p < 0.001$) and T240 ($p = 0.001$ and $p = 0.003$, respectively). The iAUCs for serum apoB48 were not different between the three meals (Table 5).

The diet x time interaction for changes in FFA also reached statistical significance (Figure 3). Overall, plasma FFA concentrations decreased during the postprandial period, but less pronounced after the HF meal as compared with the LF and LF-TB meals at T180 ($p = 0.002$ and $p = 0.005$, respectively) and T240 ($p = 0.010$ and $p = 0.026$, respectively). As shown in Table 5, the dAUC for plasma FFA tended to be smaller after the HF compared with LF meal ($p = 0.097$), whereas the dAUC after the HF meal was lower than after the LF-TB meal ($p = 0.021$).

Table 5 *iAUC and dAUC and p-values for TAG, apoB48, FFA, glucose and insulin till T240 or T120 after LF, HF or LF-TB consumption.*

	Time	HF	LF	LF-TB	Friedman	P-value		
	(min)				Sig	HF vs LF	HF vs LF-TB	LF vs LF-TB
TAG (mmol x min/L)	0-240	104.5 (18.3-170.2)	21.6 (0.0-80.2)	18.8 (10.0-64.0)	0.032*	0.038*	0.011*	0.767
ApoB48 (ng x 10^5 x min/ml)	0-240	8.8 (3.2-10.8)	10.6 (7.7-17.4)	10.6 (7.1-11.7)	0.121			
FFA (mmol x 10^4 x min/l)	0-240	4.2 (2.3-13.5)	6.9 (3.3-16.0)	6.4 (3.9-13.2)	0.050*	0.086#	0.021*	0.953
Glucose (mmol x min/l)	0-240	15.1 (8.8-122.7)	50.0 (19.2-163.6)	72.4 (8.5-130.0)	0.074			
Glucose (mmol x min/l)	0-120	15.1 (0.5-114.0)	50.0 (19.2-163.6)	78.9 (8.5-130.0)	0.016*	0.066	0.015*	0.314
Insulin (mU x 10^3 x min/ml)	0-240	3.0 (1.5-11.8)	5.6 (4.2-19.7)	8.5 (3.6-24.0)	<0.001*	0.008*	0.008*	0.021*
Insulin (mU x 10^3 x min/ml)	0-120	2.0 (1.5-10.1)	4.9 (3.3-14.8)	5.9 (3.0-17.0)	0.001*	0.008*	0.008*	0.314

Data are reported as medians with ranges, n=9.

*The Friedman test was conducted to determine significance between the diets. When the Friedman test was significant, a Wilcoxon signed rank test was performed to compare the diets pairwise. Significantly different * p<0.05*

Abbreviations: TAG: triacylglycerol, apoB48: apolipoprotein B48, FFA: free fatty acids, LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine.

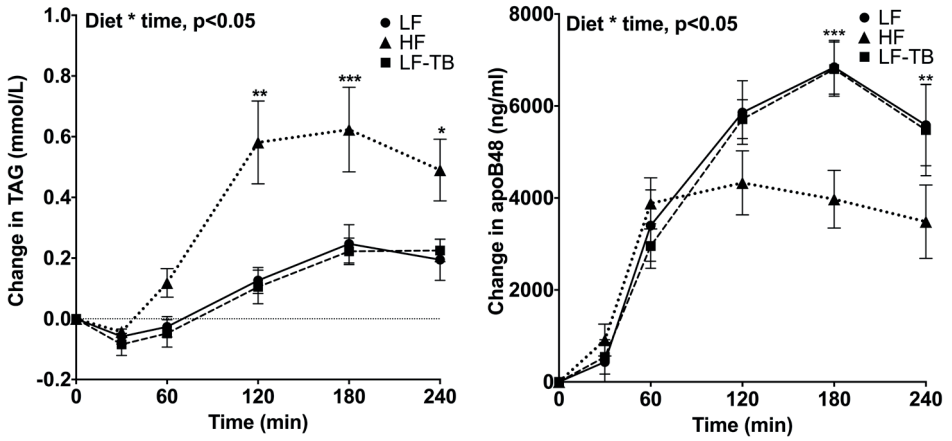

Figure 3 *A. Mean changes in serum TAG concentrations after LF, HF or LF-TB consumption. B. Mean changes in serum apoB48 concentrations after LF, HF or LF-TB consumption.*
Data are reported as mean changes ± SE, n=9.
*Linear mixed model procedures were conducted to determine significance between the diets, significantly different from the HF diet * (p<0.05), ** (p<0.01), *** (p<0.001). Abbreviations: TAG: triacylglycerol, LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine, apoB48: apolipoprotein B48*

The diet x time interaction for changes in glucose reached statistical significance (Figure 4). Increases in concentrations were lower after the HF meal as compared with the LF and LF-TB meals at T15 ($p = 0.007$ and $p = 0.004$, respectively) and T30 ($p = 0.006$ and $p = 0.003$, respectively) and were higher at T180 ($p = 0.006$ and $p = 0.003$, respectively). The iAUCs till T120 was higher after the LF-TB meal compared with the HF meal ($p = 0.015$) and tended to be higher after the LF meal compared with the HF meal ($p = 0.066$). However, the iAUCs till T240 did not differ between the three diets (Table 5). Changes in insulin showed a significant diet and time effect. Postprandial changes in plasma insulin concentrations were lower after the HF meal when compared with the LF and LF-TB meals ($p = 0.046$ and $p < 0.001$, respectively). After meal consumption changes in insulin concentrations were significantly increased at T15-T120 from baseline ($p < 0.05$ at all time points) (Figure 4). The iAUCs till T240 and T120 were both higher after the LF and LF-TB meal compared with the HF meal (all $p = 0.008$). In addition, the iAUC till T240 was greater comparing the LF-TB with the LF meal ($p = 0.021$, Table 5).

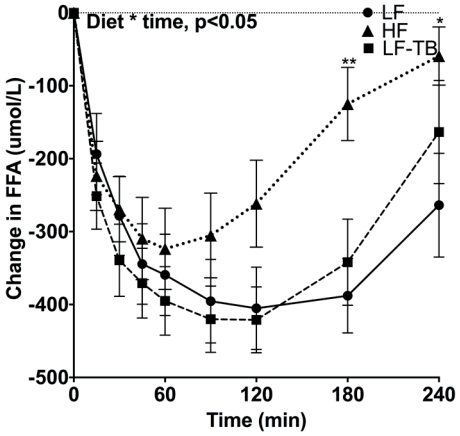

Figure 4 *Mean changes in plasma FFA concentrations after LF, HF or LF-TB consumption.*
Data are reported as mean changes ± SE, n=9.
*Linear mixed model procedures were conducted to determine significance between the diets, significantly different from the HF diet * (p<0.05), ** (p<0.01).*
Abbreviations: FFA: free fatty acid, LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine.

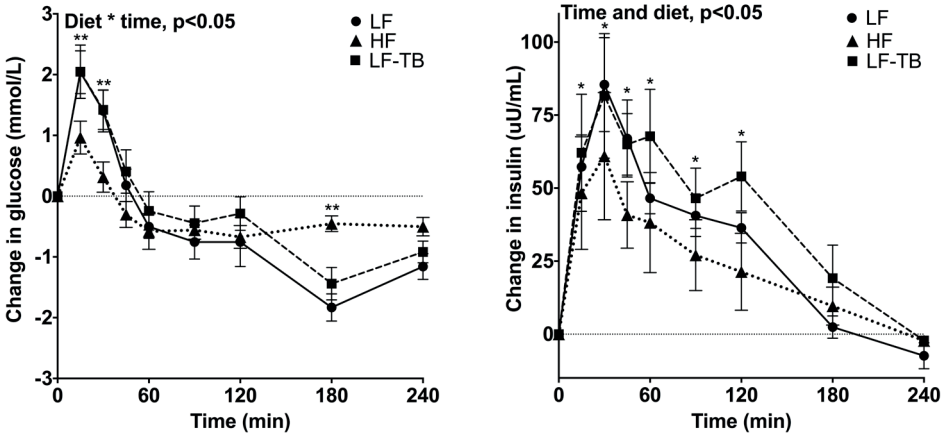

Figure 5 *A. Mean changes in plasma glucose concentrations after LF, HF or LF-TB consumption. B. Mean changes in serum insulin concentrations after LF, HF or LF-TB consumption.*
Data are reported as mean changes ± SE, n=9.
*Linear mixed model procedures were conducted to determine significance between the diets, significantly different from baseline * (p<0.05), Significantly different from the HF diet ** (p<0.01).*
Abbreviations: LF: low-fat, HF: high-fat, LF-TB: low-fat with 850 mg theobromine.

Discussion

Our hypothesis that both a HF diet and dietary theobromine increased postprandial apoA-I concentrations, when compared with a LF diet was rejected. However, we found that consumption of a HF meal showed a more pronounced increase in postprandial serum TAG concentrations as compared with a LF meal. Increased appearance of dietary fats incorporated into chylomicrons in the circulation is a normal physiologic response after a meal. Unexpectedly, the postprandial increase in apoB48 concentrations was less after intake of the HF meal as compared with the LF meal. This suggests the formation of less, but larger TAG-rich chylomicrons after HF meal consumption. Further, we expected congruent TAG and apoB48 responses during the postprandial phase, but the increase in serum apoB48 concentrations preceded the serum TAG response during the first 30 min of the postprandial phase after all meals. As expected, postprandial glucose and insulin responses were lower comparing HF with LF meal consumption, and postprandial FFA responses were less reduced comparing HF with LF meal consumption. When 850 mg of theobromine was added to a LF meal, there were no apparent changes in postprandial lipid and apolipoprotein responses. However, a significantly higher iAUC was observed for insulin concentrations after the addition of theobromine to the LF meal suggesting that more insulin was needed for maintaining glucose homeostasis.

The higher postprandial serum TAG concentrations and lower postprandial plasma glucose and insulin concentrations after the HF meal reflect the higher amount of fat and lower amount of carbohydrates in this meal. These findings are in line with those of earlier studies.[8,9,10,18]

Interestingly, after both the HF and LF meal apoB48 concentrations increased already within 30 min after meal consumption, while serum TAGs were not yet increased at this time point. For this, there are at least two explanations. First, the very first chylomicrons, which are already released before a meal enters the small intestine[19] and appear in the blood after food intake, contain pre-synthesized apoB48 proteins together with stored lipids from the previous meal.[20,21] Although in our study subjects consumed a low fat meal the evening before the three test days, it may be possible that the pre-synthesized apoB48 particles still contained small amounts of TAG, already present within the enterocyte. Why enterocytes synthesize apoB48 proteins when there are no or relatively low amounts of TAGs in the enterocytes is unknown. A second potential explanation for the absent increase in serum TAG concentrations during the first 30 minutes after the meal, while apoB48 concentrations already increased can be due to a postprandial decrease in very low density lipoprotein (VLDL) TAG concentrations.[21,22] The postprandial increase in insulin lowers VLDL production via

targeting apoB100 for degradation[23] and limiting apoB100 synthesis.[24] This decrease in VLDL TAG concentrations can counterbalance the increase in chylomicron TAG concentrations and explain the early peak in apoB48 concentrations without a rise in serum TAG concentrations. In this respect, our results are unfortunately difficult to compare with those of other studies, as no measurements were performed 30 min after meal consumption. However, after 1 hour, increases in both TAG and apoB48 have been reported,[25,26] which agrees with our findings.

Surprisingly, significantly higher postprandial apoB48 concentrations were observed after LF consumption than after HF consumption. Tremblay et al. observed a similar phenomenon after 3 days LF consumption compared with HF consumption.[27] The higher increase in apoB48 protein concentrations after the LF meal may be triggered by the higher level of monosaccharides in the LF meal, as intraduodenal infusion of a lipid and glucose emulsion increased postprandial apoB48 concentrations more as compared with infusing a lipid emulsion only.[28] Furthermore, consumption of fat with fructose or glucose significantly increased postprandial apoB48 concentrations as compared with consuming fat only.[29,30] Moreover, fast available carbohydrates increased postprandial apoB48 responses as compared with slow available carbohydrates.[25] This would imply that fat together with carbohydrate intake rather than fat intake alone fuels apoB48 formation. As we consume in daily practice not solely fat, these observations deserves further attention, since it suggests that the ratio of dietary fat to carbohydrates and the type of carbohydrates regulate intestinal chylomicron production and as such determine the type of chylomicrons that are produced.

Chylomicrons are heterogeneous particles that can vary in size according to the rate of fat absorption and the type and amount of fat absorbed, as shown in rabbits and dogs.[31] Our data now suggests that after a HF meal the lower numbers of chylomicrons are larger and contain more TAG than after a LF meal. During postprandial lipid metabolism[32,33] these larger chylomicrons are transformed into small, dense chylomicron remnants. Although it is well accepted that small dense LDL particles are more atherogenic than larger LDL particles,[34] the atherogenic capacity of small dense chylomicron remnants versus less dense chylomicron remnants is not known. On the other hand, after the LF meal a larger increase in apoB48 is observed which means that more but smaller chylomicron particles are formed. This leads to a larger number of chylomicron remnants, which might increase the risk for atherosclerosis.[35] It is not known which type of chylomicron particle, fewer and larger or more and smaller, are worse. Furthermore, in this study we only examined the acute effects

after high- or low-fat meal consumption and it is not known how these findings translate to changes after chronic high- or low-fat consumption.

Reductions in postprandial FFA concentrations were significantly more pronounced after LF meal consumption as compared with HF meal consumption. This finding is in line with other studies[36] and can be explained by the higher amount of monosaccharides in the LF shake, which causes higher glucose and consequently higher insulin concentrations. Insulin activates LPL on the endothelium of adipose tissue causing an increased TAG storage in the adipose tissue. Furthermore, insulin inhibits TAG lipolysis, leading to a lower appearance of FFA in the blood.[37] This explains the more pronounced decrease in FFA seen after LF meal consumption.

Finally, no difference in apoA-I or apoB100 concentrations was found after HF and LF meal consumption. This supports the findings of Khoury et al. who also showed no postprandial variations in apoA-I concentrations after high-fat meal consumption compared with protein or carbohydrate consumption.[38]

It has been shown that 4 weeks of theobromine consumption increased fasting serum HDL-C and apoA-I concentrations and decreased those of LDL-C and apoB.[12] However, this is the first human study that examined the acute postprandial effects of theobromine. As the intestine is a major source for apoA-I production, we were particularly interested to see if acute theobromine intake would increase postprandial apoA-I concentrations against a LF background. A LF background diet was chosen, as high-fat diets already increase fasting apoA-I concentrations.[26] However, postprandial apoA-I, apoB100, apoB48, TAG and FFA did not change after acute theobromine consumption. For these differences in acute postprandial and chronic fasting effects of theobromine are at least three possible explanations. First, it is possible that theobromine only has beneficial effects after longer-term consumption. Second, we measured the postprandial response for 4 hours, which may have been too short. Theobromine is effectively absorbed in the intestine[39] and peak plasma concentrations are usually seen 3 hours after theobromine consumption. After absorption, theobromine is transported to the liver where it is metabolized. Peak concentrations of its main metabolites, 7-methylxanthine and 3-methylxanthine, are found 3 to 7.5 hours after theobromine consumption.[40] In our study, blood was sampled up to 4 hours after meal consumption. If the metabolites of theobromine are responsible for the effects seen in the fasting state, it is possible that we may have missed such effects in our 4-hour postprandial study. Third, it is also possible that theobromine does not change postprandial lipid metabolism at all.

Glucose and insulin both showed a small non-significant increase after 45 min of LF-TB consumption compared with LF meal consumption. Furthermore the iAUC of insulin till T240 was larger after the LF-TB meal compared with the LF meal. Whether

this indicates a decrease in insulin sensitivity after theobromine consumption needs further study.

Our study has several limitations. First, only healthy men were studied and more research is needed to conclude if our results can be extrapolated to women or less healthy population groups. Moreover, we performed an acute study and it cannot be excluded that more or different pronounced postprandial effects of theobromine and dietary fat are evident after longer-term intakes. Finally, we did deliberately not include a HF-TB treatment, as we wanted to minimize the possibility that the dietary fat content of the meal masked the potential theobromine effect on apoA-I. However, it cannot be excluded that theobromine effects are changed by the amount of fat in the diet.

In healthy men, HF meal consumption induced lower postprandial apoB48 responses as compared to LF meal consumption. Together with a higher TAG response, this suggests the formation of less, but larger chylomicrons. Whether this translates into the formation of potentially atherogenic small dense chylomicron remnants needs further study. In addition, acute theobromine consumption did not affect postprandial lipid metabolism. Furthermore, acute HF or theobromine consumption did not affect postprandial apoA-I concentrations.

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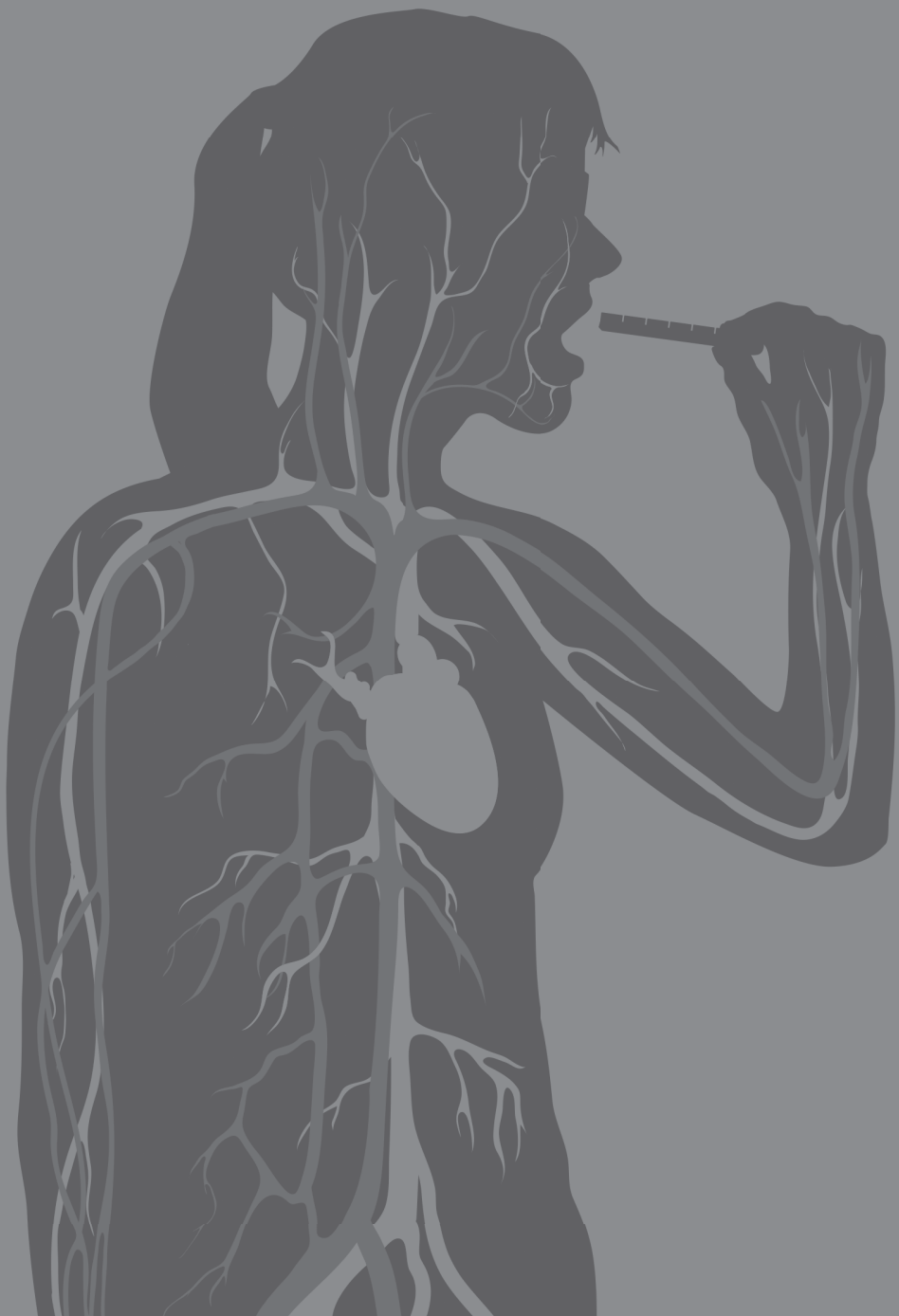

Chapter 4

The acute effects of dietary theobromine and fat on duodenal gene expression in healthy men

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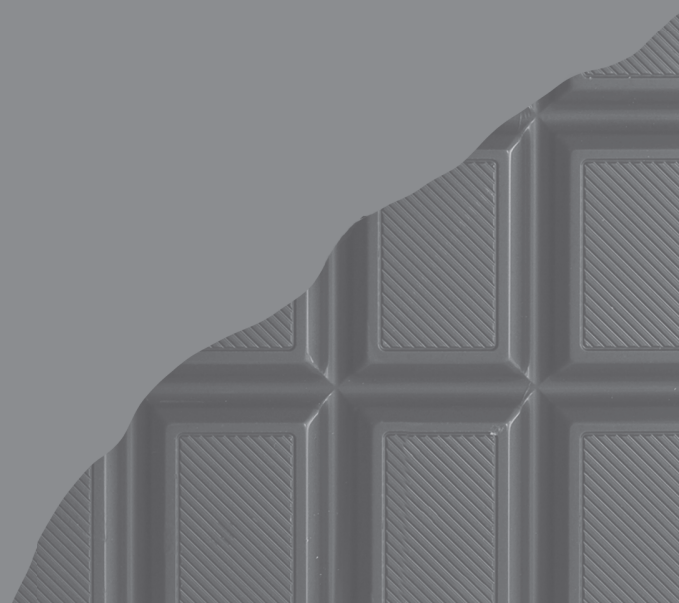
To be submitted



Abstract

Background and aim Increasing apolipoprotein A-I (apoA-I) synthesis may improve high-density lipoprotein (HDL) functionality and lower cardio vascular disease (CVD) risk. As theobromine and fat have been reported to increase fasting serum apoA-I concentrations, and the intestine is involved in apoA-I production, the acute effects of theobromine and fat on gene transcription in the duodenum were studied to better understand underlying mechanisms.

Material and methods In this crossover study, 8 healthy men received in random order once a low fat (LF) meal as control, the LF meal plus theobromine (850 mg), or a high fat (HF) meal. Before and after meal intake, blood samples were taken for the analysis of inflammation markers, endothelial markers and intestinal fatty acid-binding protein (IFABP). Five hours after meal intake duodenal biopsies were taken for microarray analysis.

Results Theobromine and HF meal consumption did not change duodenal apoA-I mRNA expression. Adding theobromine to a LF meal did not change the expression of genes related to lipid and cholesterol metabolism, whereas those related to glycogen and glucose breakdown were downregulated. HF consumption increased the expression of genes related to lipid and cholesterol uptake and transport, and to glucose storage, while it decreased those related to glucose uptake. Furthermore, genes related to inflammation were upregulated, but plasma inflammation markers and IFAPB were not changed.

Conclusion In healthy men, acute theobromine and fat consumption did not change apoA-I expression in the duodenum. Both theobromine and HF consumption inhibited gene expression related to the glucose metabolism. Furthermore, HF intake activated in the duodenum the expression of genes related to lipid and cholesterol metabolism and inflammation.

Introduction

Over the past decade, interventions aiming to increase serum high-density lipoprotein (HDL) cholesterol (HDL-C) concentrations to reduce the risk of cardiovascular disease (CVD) have not been successful. Recent insights, however, suggest that improving HDL functionality will more likely lower CVD risk than simply elevating circulating serum HDL-C concentrations.[1] In this respect, increasing serum apolipoprotein A-I (apoA-I) synthesis may be a promising approach, as serum apoA-I concentrations correlate with *in vitro* cholesterol efflux, a measure of HDL functionality.[2-4] For this, various pharmaceutical approaches are currently explored.[5] However, as a preventive strategy at a population level, dietary strategies are more useful. Unfortunately, the number of (novel) dietary components that increase apoA-I production is limited. Theobromine, a component from cocoa, has been reported to increase fasting serum apoA-I concentrations.[6] Mechanisms explaining the effects of theobromine on fasting serum apoA-I concentrations are however not clear. Since apoA-I is produced in enterocytes and hepatocytes,[7] and theobromine is absorbed in the small intestine,[8,9] we were interested in exploring the effects of acute theobromine consumption on gene expression in postprandial human duodenal biopsies, following theobromine intake. Duodenal biopsies were used because apoA-I secretion is higher in the duodenum as compared with other parts of the intestine.[10]

Except theobromine, also exchanging carbohydrates for fatty acids increases fasting serum apoA-I concentrations.[11,12] Although the intestine is strongly involved in dietary lipid handling, the effects of fat intake on duodenal gene expression profiles have not been studied. Only a limited number of studies compared the effects of a low-fat/high-carbohydrate (LF) with a high-fat/low-carbohydrate (HF) meal on gene expression profiles, and so far only in human muscle biopsies[13-15] and peripheral blood mononuclear cells (PBMCs).[16] We therefore used a nutrigenomic approach to analyze differences in gene expression in human duodenal biopsies after adding 850 mg of theobromine to a LF meal, and after comparing HF with LF meal consumption to better understand effects of dietary theobromine and fat on duodenal apoA-I transcription and related pathways.

Material and methods

Study population and design

The design and results of the metabolic parameters of this double-blind crossover study have already been reported.[17] Briefly, ten apparently healthy men participated. During the screening visit, body weight, height and blood pressure were measured

and a fasting blood sample was taken. Subjects were excluded when fasting serum total cholesterol concentrations ≥8.0 mmol/L or plasma glucose concentrations ≥7.0 mmol/L. After inclusion, all subjects participated in three test days, each separated by a one-week washout period. Two weeks before the start of the study, subjects were instructed to avoid products containing cocoa till the end of the study period. Also, the consumption of caffeine containing drinks was restricted to a maximum of 4 cups a day, since theobromine is a metabolite of caffeine. The study was conducted according the guidelines laid down in the Declaration of Helsinki. The study protocol was approved by the Medical Ethical Committee of the University Hospital Maastricht. All participants gave their written informed consent before entering the study. The study was registered on clinicaltrials.gov under study number NCT02085109.

Test days

To minimize differences in dietary intake before the three test days, all subjects were provided with a standard low fat dinner the evening before each test day, which consisted of a commercially available macaroni, 3 crackers, and a dairy drink. The next morning, subjects came to the University in fasting condition, which means that after dinner the preceding evening, they had not consumed any foods or drinks, except for water. To reduce physical activity as much as possible, participants arrived by public transport or car on the morning of the test day. After a 15 min rest, the first fasting blood sample was collected (T0) via an intravenous cannula inserted into the antecubital vein. Subjects were then asked to consume a shake within 10 min. Three different shakes were provided in random order. One shake was low-fat/high-carbohydrate (LF), one shake was LF enriched with 850 mg of theobromine (LF-TB), and one shake was high-fat/low-carbohydrate (HF) (Table 1). The theobromine powder (Fagron, Uitgeest, the Netherlands) was added as the final ingredient to the blender jar before it was thoroughly mixed with the LF shake. The volumes of the shakes were standardized with water. The difference between the HF and the LF shakes was an exchange between fat and carbohydrates, while the amount of proteins between the three shakes was comparable (Table 1).

Shakes were prepared by the research dietician to blind both researchers and participants. After consumption of the shakes, the volunteers were not allowed to eat or drink anything except water for the next 5 hours. Other blood samples were taken at T = 30 (T30), T = 60 (T60), T = 90 (T90), T = 120 (T120), T = 180 (T180) and T = 240 (T240) min. Five hours after meal intake, duodenal biopsies were taken at the Department of Endoscopy. During duodenoscopy, no sedatives were given. Four duodenal mucosal tissue samples, just proximal of the ampulla of Vater, were taken using standard biopsy forceps. The diameter of the biopsies varied between 2.0 mm

and 2.2 mm. After sampling, biopsies were immediately frozen in liquid nitrogen, stored at -80˚C and analyzed at the end of the study for gene expression profiles.

Table 1 *Nutrient composition of the low-fat/high-carbohydrate (LF), LF with 850 mg theobromine (LF-TB) and high-fat/low-carbohydrate (HF) shakes*

Nutrient	LF	LF-TB	HF
Energy (kcal)	956	956	965
Protein (g)	19.4	19.4	17.9
(E%)[1]	8	8	7
Carbohydrates (g)	193.7	193.7	85.7
(E%)	81	81	35
Mono- and disaccharides (g)	144.9	144.9	45.6
Polysaccharides (g)	48.8	48.8	40.1
Total fat (g)	10.5	10.5	60.6
(E%)	10	10	56
Saturated fatty acids (g)	3.2	3.2	36.0
Monounsaturated fatty acids (g)	4.0	4.0	18.7
Polyunsaturated fatty acids (g)	1.1	1.1	4.1
Cholesterol (mg)	334	334	341
Theobromine (mg)	0	850	0

[1] *E%: energy percent*

Blood sampling and analysis

Blood was sampled in serum and EDTA-containing vacutainer tubes. Serum tubes were allowed to clot for 1 hour at 20˚C, followed by centrifugation at 1300 x g for 15 min at 20˚C. The EDTA tubes were placed on ice directly after sampling and centrifuged at 1300 x g for 15 min at 4˚C within 60 min after sampling. Serum and plasma aliquots were stored at -80˚C until analyses. All samples from one subject were analyzed within the same analytical run at the end of the study.

Because gene expression profiles clearly showed a more pro-inflammatory pattern after HF compared with LF consumption, concentrations of the inflammation and acute phase response markers: interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), serum amyloid A (SAA), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), and high sensitive C-reactive protein (hsCRP), were measured in plasma samples of the HF and LF test days (T0, T90 and T240). In addition we analyzed plasma concentrations of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) to evaluate a possible cross-talk between an "inflamed" intestine and the vascular wall. For these analyses, a commercially

available Multi Spot ELISA kit (Meso Scale Discovery, Rockville, MD, USA) was used. Finally, in the same plasma samples, intestinal fatty acid-binding protein (IFABP) concentrations, which is a marker for damaged enterocytes,[18,19] were measured using a sandwich ELISA (R&D, Oxon, United Kingdom) to evaluate whether one acute HF meal can already damage the enterocytes.

Microarray processing and data analysis

Total RNA was extracted from one frozen duodenal mucosal biopsy using TRIzol reagent (Invitrogen, Breda, the Netherlands) and purified on columns using Qiagen RNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Total RNA (100 ng) was labeled by Whole Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19697 unique genes (Affymetrix, Santa Clara, CA). Microarray analyses were performed using MADMAX pipeline for statistical analysis of microarray data.[20] In short, microarrays were normalized with the robust multichip average method and probes were annotated as described.[21,22] This gene set was filtered on an expression of >10 on at least 5 arrays and measured with ≥5 probes. This filtered data set consisted of 10506 genes. Comparisons were made between the LF-TB and the LF meal and between the HF and the LF meal. Individual genes were defined as changed when comparison of the normalized signal intensities showed a $p \leq 0.05$ in a 2-tailed paired intensity-based moderated t-statistics (IBMT) and a fold change of >1.2 or <-1.2 between the diets.[23] Further data analysis was performed on the filtered dataset with three different approaches i.e. Ingenuity Pathway Analysis (IPA), Upstream Regulator Analysis and Gene Set Enrichment Analysis (GSEA).[24] Pathways were selected on a –log p-values of ≤1.3, which indicates a significant change of $p \geq 0.05$ in that specific pathway comparing the LF-TB with LF diet or the HF with the LF diet. In the Upstream Regulator Analysis, Ingenuity software uses a curated database of interactions on the basis of the literature to link significant gene sets with upstream regulators. Significant linked gene sets were selected using a p-value of <0.05 for gene expression and a p-value of overlap of <0.05. A z-score above 1.5 indicates activation, whereas a z-score below -1.5 indicates inhibition of this upstream regulator. GSEA was performed on the unfiltered data set; gene sets were selected on a False Discovery Rate (FDR) q-value of <0.2 and were ranked on the Normalized Enrichment Score (NES). During microarray analysis we were especially interested in expression changes in apoA-I transcription and related pathways including the lipid, cholesterol and glucose metabolism, inflammation and the immune system.

Statistical analysis

All data are presented as mean ± SD unless otherwise indicated. Differences in changes of inflammatory markers, endothelial function markers and IFABP between the HF and LF meal were evaluated with general mixed models with subject as random factor, diet and time as fixed factors and a diet*time interaction. If this diet*time interaction was not significant, it was omitted from the model. If the factor time was significant, time points were compared to baseline concentrations, using Bonferroni's corrections for multiple comparisons. Results were considered to be statistically significant if $p \leq 0.05$. All statistical analyses were performed using SPSS 20.0 for Mac (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

All ten men completed the study. However, results of one man were excluded due to protocol violation, as he appeared not to be in fasting condition at start of one of the test days. Samples from a second subject were excluded due to technical issues during microarray analysis. Baseline characteristics of the final eight subjects are shown in Table 2.

Table 2 *Baseline characteristics of the participants who completed the study (n = 8).*

	Mean ± SD
Age (years)	38 ± 15
BMI (kg/m^2)	24.3 ± 2.0
Serum total cholesterol (mmol/L)	5.2 ± 0.9
Plasma glucose (mmol/L)	5.1 ± 0.3
Systolic blood pressure (mmHg)	130 ± 18
Diastolic blood pressure (mmHg)	79 ± 14

Microarray analysis

From the 19697 genes present on the microarray, 10506 genes were expressed in the duodenum (expression value >20 and >5 probes per gene on the array). In comparison to the LF shake, 113 and 286 genes were differentially expressed after adding theobromine to the LF diet (LF-TB) and comparing it with the HF shake, respectively (Supplementary data Figure 1). Twenty-three of these differentially expressed genes overlapped, i.e. the expression was significantly changed into the same direction after both the LF-TB and the HF interventions as compared with the

LF diet. These 23 genes were SCN3B, PCDH11Y, NELL2, LYPD6B, ZNF485, FBXL16, PLA1A, ZBTB16, UPK2, DKK4, TIAM2, CKLF, SPRR1A, C12orf74, MTNR1A, KCNS3, MBOAT2, VSIG8, TEN1-CDK3, PDE10A, LRP12, TAS2R3 and RAD51AP1. So far, none of these 23 genes was described in relation to apoA-I transcriptional regulation. Unfortunately, apoA-I gene expression did not change when comparing the LF-TB meal vs. the LF meal (FC = 1.02, P = 0.758) or the HF meal vs. the LF meal (FC = 1.07, P = 0.310). Still, these 23 genes are potentially interesting targets to consider in the context of apoA-I transcription.

Next, gene expression profiles were further explored, to better understand the effects of adding theobromine to a LF diet or comparing a HF with a LF diet on duodenal gene expression. First, IPA was used to look at significantly changed pathways. Then, two more in depth analytical procedures were conducted, i.e. the Upstream Regulator Analysis and GSEA.

Adding TB to a LF meal

Ingenuity Pathway Analysis

Thirty pathways were differentially regulated by theobromine consumption (Figure 1). None of the pathways was related to cholesterol, lipid or glucose metabolism.

Upstream Regulator Analysis

Adding TB to the LF meal changed the activation of 29 transcriptional regulators (Supplementary data, Table 1). Three of these upstream regulators, which were related to glucose metabolism, were inhibited. Further, the other identified upstream regulators were not linked to the lipid and cholesterol metabolism (Table 3).

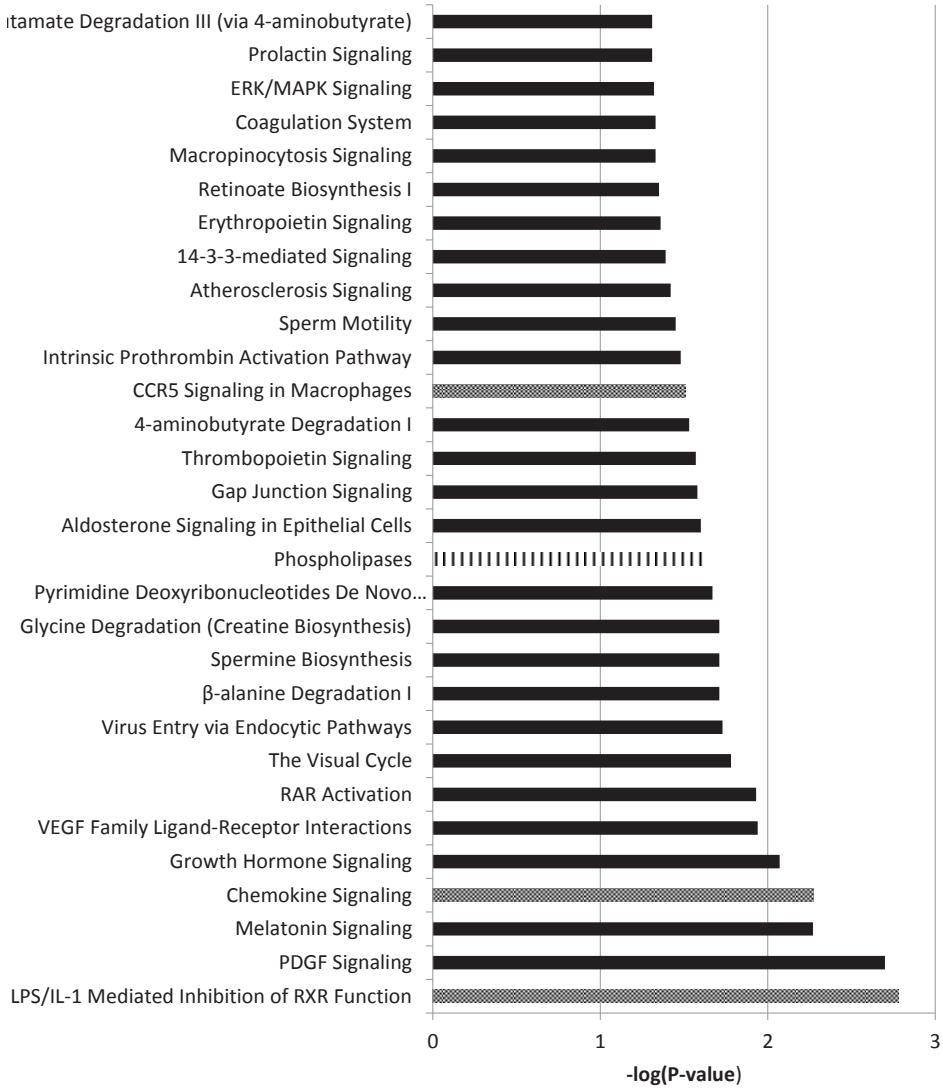


Figure 1 *Significantly different pathways after adding 850 mg of theobromine (TB) to the low-fat/high-carbohydrate (LF) diet. Dotted bars are pathways involved in the immune system, lined bars are pathways involved in cholesterol, lipid or glucose metabolism (n = 8)*

Table 3 *Inhibited or activated upstream regulators after adding 850 mg of theobromine (TB) to a low-fat/high-carbohydrate (LF) meal or after comparing high-fat/low-carbohydrate (HF) with LF consumption functioning in lipid, cholesterol or glucose metabolism or the immune system (n = 8)*

Upstream regulator	Activation Z-score	Function (gene card ref)	Comparison
Ins1	-3.02	Decreases blood glucose	LF-TB vs. LF
Insulin	-2.08	Decreases blood glucose	LF-TB vs. LF
INS	-1.95	Decreases blood glucose	LF-TB vs. LF
INSIG2	-1.95	Feedback control of cholesterol synthesis	HF vs. LF
APOE	-1.73	Main lipoprotein on chylomicrons	HF vs. LF
ACOX1	-1.63	Fatty acid β-oxidation pathway	HF vs. LF
SREBF2	1.93	Lipid homeostasis	HF vs. LF
PPARα	1.97	Transcription factor in lipid and cholesterol metabolism	HF vs. LF
FABP2	2.00	Uptake and transport of long chain fatty acids involved in TAG-rich lipoprotein synthesis	HF vs. LF
SREBF1	2.27	Transcription factor which regulates lipid homeostasis	HF vs. LF
GCG	-2.42	Proprotein for glucagon	HF vs. LF
GCK	-1.98	Enzyme functioning in glucose utilization	HF vs. LF
Gsk3	1.98	Glycogen synthesis	HF vs. LF
TNFSF12	1.65	Activation of NFκB, inducer of proinflammatory cytokines	HF vs. LF
IL12	1.90	Proinflammatory cytokine	HF vs. LF
IL2	1.92	Proinflammatory cytokine	HF vs. LF
CD5	1.98	Receptor in the regulation of T-lymphocyte proliferation	HF vs. LF
CCL5	1.98	Chemoattractant for monocytes, T-lymphocytes, eosinophils	HF vs. LF
TNF	1.99	Survival, proliferation and differentiation of monocytes and macrophages	HF vs. LF
RELA	2.02	Subunit NFκB	HF vs. LF
MYD88	2.24	Innate immune response	HF vs. LF
IL1A	2.31	Proinflammatory cytokine	HF vs. LF
TNFSF11	2.73	T-lymphocyte dependent immune response	HF vs. LF
IL1B	2.82	Proinflammatory cytokine	HF vs. LF

Gene Set Enrichment Analysis

In the GSEA, 6 gene sets were upregulated and 2 gene sets were downregulated (Supplementary data, Table 2). One of the downregulated set was glucose metabolism (Table 4).

Table 4 *Significantly inhibited or activated gene sets after adding 850 mg of theobromine (TB) to a low-fat/high-carbohydrate (LF) meal or after comparing high-fat/low-carbohydrate (HF) with LF meal consumption functioning in lipid, cholesterol or glucose metabolism or the immune system (n = 8)*

Name gene set	NES	FDR q-val	Comparison
Glucose metabolism	-1.96	0.137	LF-TB vs. LF
Regulation of beta cell development	1.96	0.119	LF-TB vs. LF
Fatty acid beta oxidation	2.09	0.025	HF vs. LF
Regulation of lipid metabolism by PPARα	1.93	0.057	HF vs. LF
PPARα activates gene expression	1.92	0.058	HF vs. LF
Mitochondrial long chain fatty acid β oxidation	1.88	0.075	HF vs. LF
TAG synthesis	1.82	0.106	HF vs. LF
Regulation of lipid metabolism by PPARα	1.81	0.104	HF vs. LF
PPARα signaling pathway	1.80	0.104	HF vs. LF
PPARα targets	1.80	0.101	HF vs. LF
Statin pathway	1.67	0.144	HF vs. LF
SREBP signaling	1.62	0.159	HF vs. LF
Lipid digestion, mobilization and transport	1.60	0.171	HF vs. LF
Fatty acid TAG and ketone body metabolism	1.60	0.167	HF vs. LF
Lipid digestion, mobilization and transport	1.56	0.184	HF vs. LF
Metabolism of steroid hormones and Vit D	1.58	0.175	HF vs. LF
Steroid hormones	1.57	0.178	HF vs. LF
Regulation of cholesterol synthesis by SREBP SREBF	1.56	0.181	HF vs. LF
Lipid digestion, mobilization and transport	1.55	0.183	HF vs. LF
Cholesterol biosynthesis	1.53	0.199	HF vs. LF
Glucose metabolism	-2.29	0.001	HF vs. LF
Metabolism of carbohydrates	-2.17	0.006	HF vs. LF
Gluconeogenesis	-1.84	0.074	HF vs. LF
Hexose transport	-1.83	0.073	HF vs. LF
Carbohydrates digestion and absorption	-1.83	0.070	HF vs. LF
Glycogen metabolism	-1.78	0.064	HF vs. LF
Starch and sucrose metabolism	-1.77	0.058	HF vs. LF
Hexose transport	-1.67	0.088	HF vs. LF
Pancreatic secretion	-1.61	0.129	HF vs. LF
Insulin signalling pathway	-1.59	0.136	HF vs. LF
Glycolysis and gluconeogenesis	-1.47	0.193	HF vs. LF
Type II diabetes mellitus	-1.47	0.196	HF vs. LF
Glycogen storage diseases	-1.46	0.198	HF vs. LF
Glucose transport	-1.45	0.196	HF vs. LF
Chemokine receptors bind chemokines	2.08	0.020	HF vs. LF

Table 4 *Significantly inhibited or activated gene sets after adding 850 mg of theobromine (TB) to a low-fat/high-carbohydrate (LF) meal or after comparing high-fat/low-carbohydrate (HF) with LF meal consumption functioning in lipid, cholesterol or glucose metabolism or the immune system (n = 8) (continued)*

Name gene set	NES	FDR q-val	Comparison
Cell adhesion molecules CAMS	1.82	0.113	HF vs. LF
Cytokine cytokine receptor interaction	1.82	0.110	HF vs. LF
Cytokine and inflammatory response	1.80	0.103	HF vs. LF
Intestinal immune network for IGA production	1.79	0.106	HF vs. LF
RIP mediated NFkB activation via ZBP1	1.78	0.106	HF vs. LF
ZBP1 DAI mediated induction of type I IFNS	1.78	0.101	HF vs. LF
IL1R pathway	1.78	0.089	HF vs. LF
Rheumatoid arthritis	1.77	0.101	HF vs. LF
NKT pathway	1.74	0.110	HF vs. LF
TNF signaling pathway	1.73	0.112	HF vs. LF
Staphylococcus aureus infection	1.71	0.126	HF vs. LF
NFkB signaling pathway	1.65	0.176	HF vs. LF
TOPB1 pathway	1.60	0.168	HF vs. LF
NTHI pathway	1.59	0.173	HF vs. LF
Inflammatory response pathway	1.59	0.167	HF vs. LF
TNFR2 pathway	1.57	0.180	HF vs. LF
Human complement system	1.55	0.184	HF vs. LF

Overall gene expression pattern after adding TB to a LF meal

Overall, adding TB to a LF meal did not change the expression of genes related to lipid and cholesterol metabolism, whereas, expression of a number of genes related to glycogen and glucose breakdown were downregulated.

Comparing HF with LF meal consumption

Ingenuity Pathway Analysis

Fifty pathways were differentially regulated after intake of the HF meal as compared with the LF meal. Seven of these pathways were linked to cholesterol, lipid or glucose metabolism, such as LXR/RXR activation and triacylglycerol degradation/biosynthesis. Also 9 changed pathways were related to the immune response, including the production of IL12 and IL15 and the production of NO and ROS in macrophages (Figure 2).

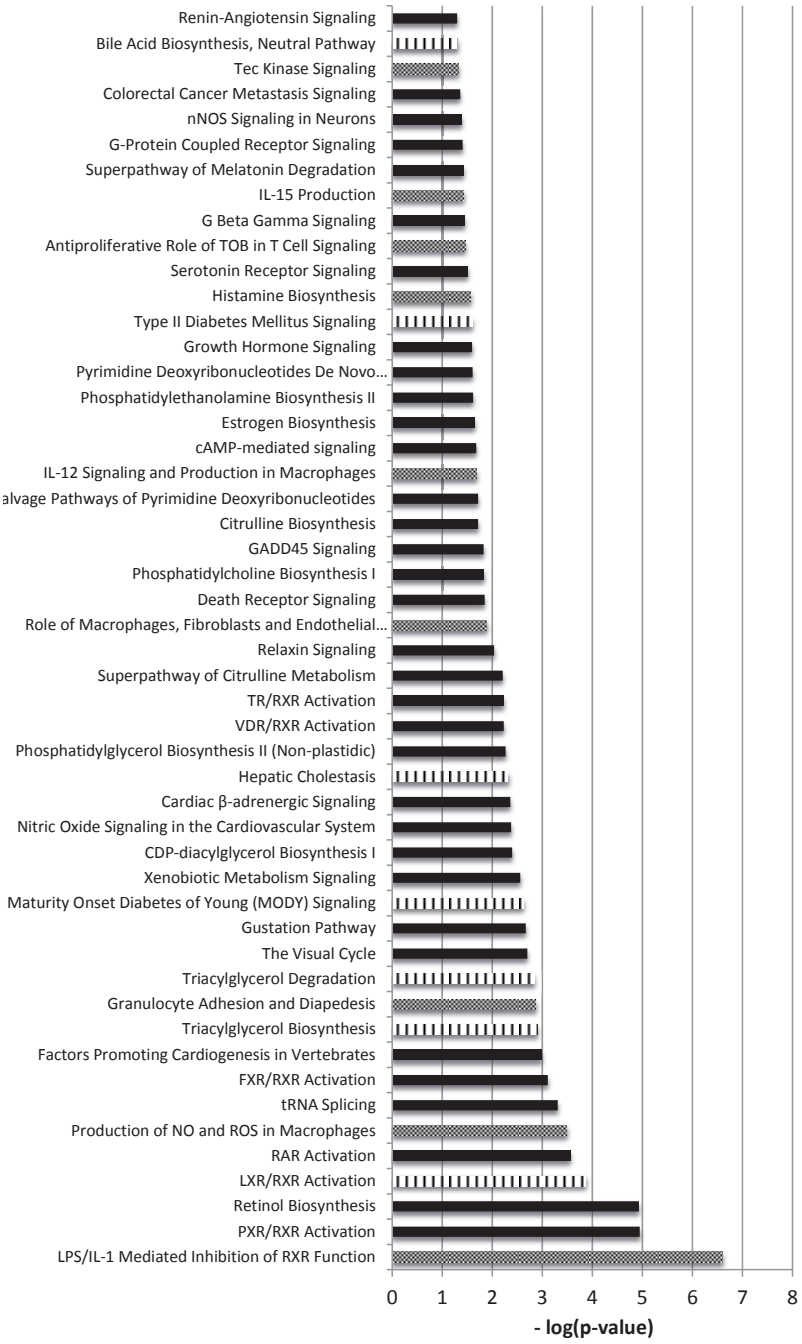


Figure 2 *Significantly different pathways comparing a high-fat/low-carbohydrate (HF) with a low-fat/high-carbohydrate (LF) diet. Dotted bars are pathways involved in the immune system, lined bars are pathways involved in cholesterol, lipid or glucose metabolism (n = 8)*

Upstream Regulator Analysis

The activation of 113 transcriptional regulators was changed (Supplementary data, Table 3). After HF consumption regulators involved in lipid uptake and transport such as SREBF2, PPARα and FABP2 were activated, while regulators involved in fatty acid breakdown such as ACOX1 were inhibited. Furthermore, regulators involved in glucose uptake, including GCG and GCK were inhibited and regulators involved in glucose storage, such as Gsk3 were activated. Finally, HF meal consumption activated 11 proinflammatory regulators involved in the immune response: TNFSF12, IL12, IL2, CCL5, TNF, CD5, RELA, MYD88, TNFSF11, IL1A and IL1B (Table 3).

Gene Set Enrichment Analysis

GSEA showed 83 upregulated gene sets and 176 downregulated gene sets after HF meal consumption (Supplementary data, Table 4). Seventeen gene sets involved in lipid and cholesterol metabolism were upregulated. These gene sets suggested an increased activity of PPARα, fatty acid oxidation, fatty acid, triacylglycerol (TAG) and lipoprotein metabolism, lipid digestion, mobilization and transport, cholesterol synthesis, steroid hormone metabolism and SREBP signaling. Furthermore, the 13 gene sets gene sets that were downregulated were involved in glucose and carbohydrate metabolism, suggesting an inhibited glucose and carbohydrate metabolism, gluconeogenesis and insulin signaling. Finally, 19 upregulated gene sets were involved in the immune system, including chemokine and cytokine activities, NFκB pathway, complement system and TNF signaling pathway (Table 4 and Figure 3).

Overall gene expression pattern comparing HF with LF consumption

HF consumption increased the expression of genes related to lipid and cholesterol uptake and transport and glucose storage, while it decreased the expression of genes related glucose uptake. Furthermore, all three approaches showed upregulated expression of genes related to the immune response and inflammation.

Markers for inflammation, the acute phase response, endothelial function and intestinal damage

Since the three analytical approaches consistently showed differences in the expression profiles of genes involved in the cytokine and inflammatory responses after HF compared with LF meal consumption, a panel of plasma markers for inflammation and acute phase responses were analyzed to evaluate whether this acute change in inflammatory gene expression in the duodenum was also transferred into the circulation.

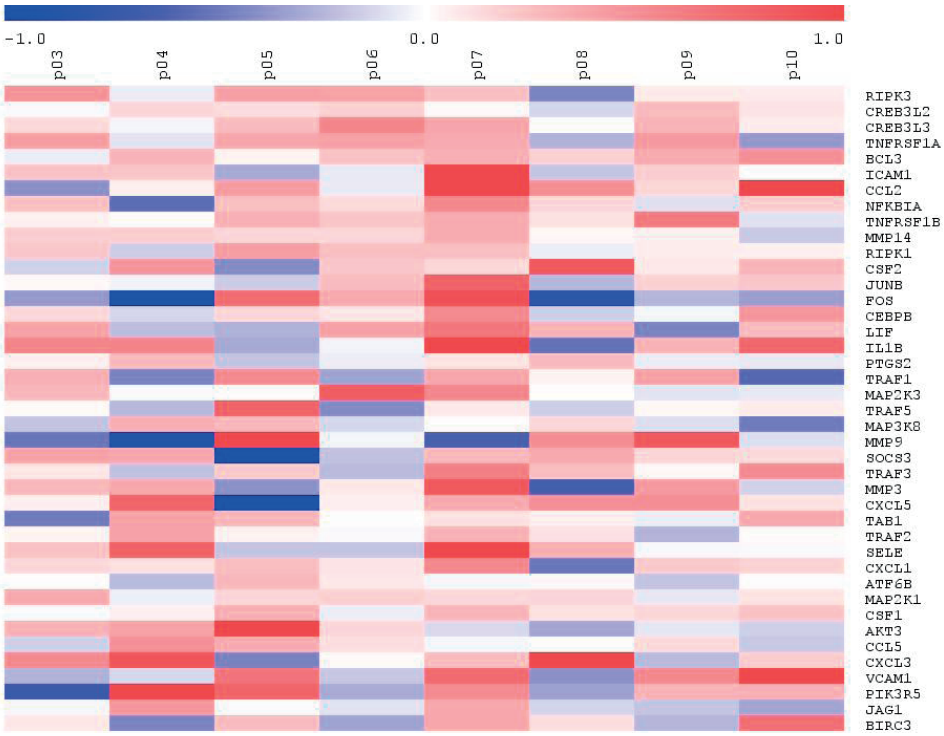


Figure 3 *Heatmap of the of the TNF signaling pathway from the GSEA results after the consumption of an acute high-fat/low carbohydrate (HF) vs. low-fat/high-carbohydrate (LF) meal (n = 8)*

During the postprandial phase, IL-6 concentrations significantly increased over time ($P < 0.001$), while IL-8, MCP-1 and IFABP concentrations significantly decreased over time ($P = 0.006$, $P < 0.001$ and $P < 0.001$, respectively). Concentrations of all other markers for inflammation and the acute phase response, as well as endothelial function did not change over time (Figure 4). Moreover, no differences in postprandial changes in markers for inflammation, acute phase response and endothelial function between the HF and LF meals were observed (Figure 4). Finally, circulating levels of IFABP were measured to evaluate the potential effects of the HF and LF meal on intestinal damage. However, also IFABP concentrations were not different between HF and LF meal consumption (Figure 5).

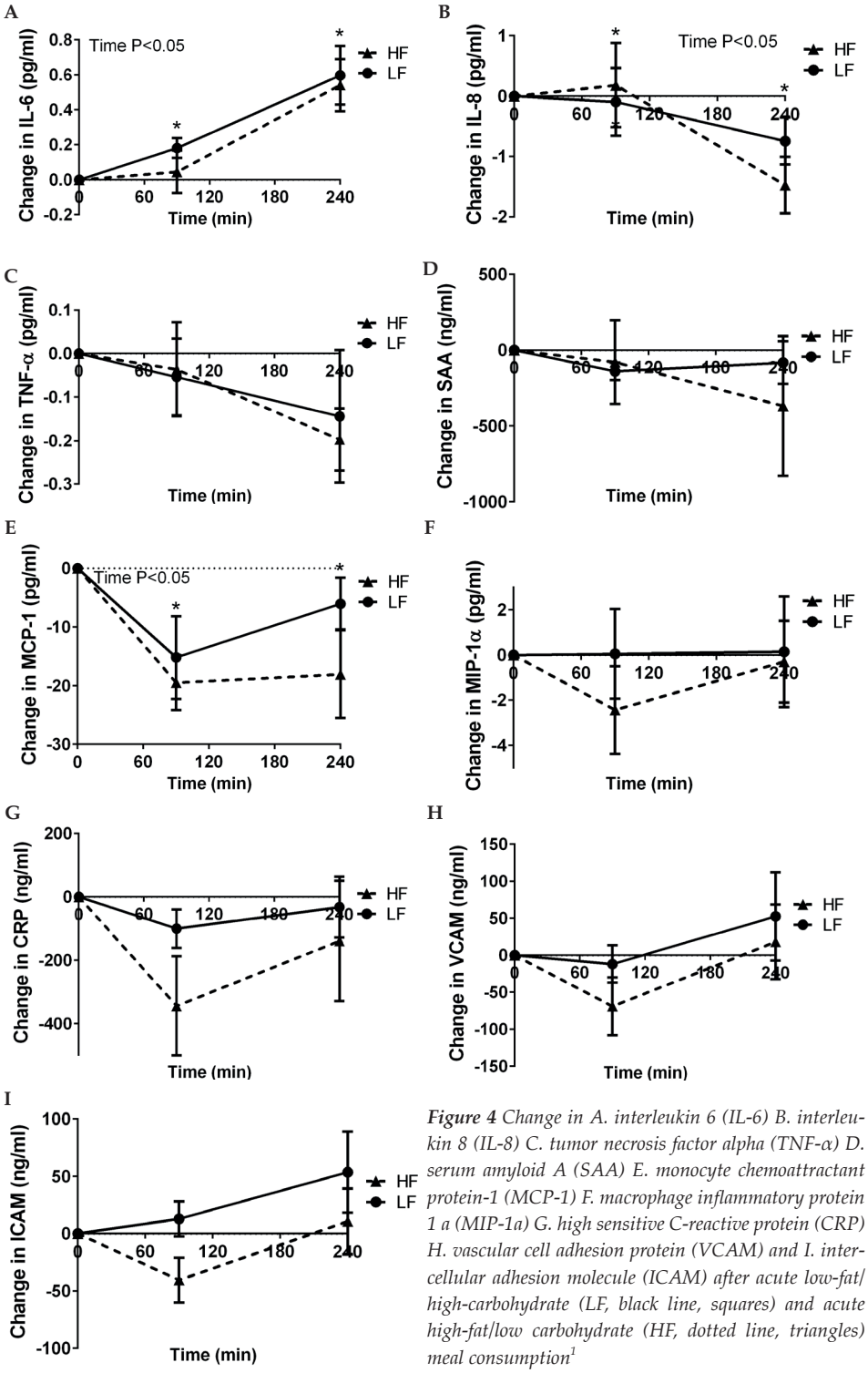


Figure 4 *Change in A. interleukin 6 (IL-6) B. interleukin 8 (IL-8) C. tumor necrosis factor alpha (TNF-α) D. serum amyloid A (SAA) E. monocyte chemoattractant protein-1 (MCP-1) F. macrophage inflammatory protein 1 a (MIP-1a) G. high sensitive C-reactive protein (CRP) H. vascular cell adhesion protein (VCAM) and I. intercellular adhesion molecule (ICAM) after acute low-fat/high-carbohydrate (LF, black line, squares) and acute high-fat/low carbohydrate (HF, dotted line, triangles) meal consumption*[1]

[1] *Values are mean ± SD. n = 8.*

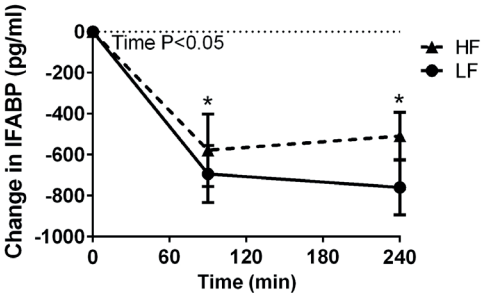


Figure 5 *Change in intestinal fatty acid-binding protein (IFABP) after acute low-fat/high-carbohydrate (LF, black line, squares) and acute high-fat/low-carbohydrate (HF, dotted line, triangles) meal consumption [1] Values are mean ± SD. n = 8.*

Discussion

Earlier it has been shown that the consumption of theobromine[6] or fat[12] increased fasting serum apoA-I concentrations. To better understand underlying mechanisms, the present randomized, double-blind, controlled study examined the acute effects of theobromine and HF consumption on apoA-I at the transcriptional level in the small intestine, an organ involved in apoA-I synthesis. Unfortunately, both theobromine and HF consumption did not change duodenal apoA-I gene expression. For these apparently discrepant findings, there are at least four explanations. First, theobromine and fat only change duodenal apoA-I mRNA expression after longer-term consumption. Second, postprandial effects on apoA-I transcription are not evident within a time period of 5 hours. Third, theobromine and fat regulate apoA-I metabolism not at a transcriptional level in the duodenum, but only in the liver. However, this explanation is less likely, as postprandial serum apoA-I concentrations did also not change after theobromine and HF intake.[17] Fourth, theobromine and fat do not change apoA-I transcription but increases serum apoA-I concentrations via other mechanisms, e.g. apoA-I clearance. Indeed, replacement of 13 energy % of carbohydrates for MUFA decreased apoA-I fractional catabolic rate, while it did not change apoA-I production rate.[25] It should also be noted, that unexpectedly we could recently not confirm in a long-term study the effects of theobromine on apoA-I,[26] as reported by Neufingerl et al..[6]

Furthermore, acute theobromine consumption did not change the expression of genes related to lipid and cholesterol metabolism. This is in agreement with the earlier reported acute effects on postprandial serum concentrations of apoB100, apolipoprotein B48, TAG and free fatty acids,[17] but not with the results of 4-weeks of theobromine consumption, which improved fasting lipid metabolism.[6,26] Except for apoB48, which is only synthesized in the intestine, the same explanations can be used to explain the lack of effects as those for apoA-I. Moreover, theobromine

inhibited glucose metabolism in the duodenum, suggesting a lower glycogen and glucose breakdown. In the circulation, however, we have earlier reported increased postprandial insulin responses after acute consumption,[17] and increased postprandial glucose and insulin responses after 4-week consumption.[26] Altogether, this suggests that acute theobromine consumption affects glucose metabolism, but it is unclear how the decreased gene expression related to glucose metabolism in the duodenum relates to the increased insulin concentrations in the circulation.

Surprisingly, the small intestine is the least studied organ involved in lipid homeostasis, although it plays a major role in TAG and cholesterol absorption and transport through the formation of chylomicron particles.[27] As expected, the HF meal increased postprandial serum TAG concentrations,[17] and upregulated genes related to lipid and cholesterol uptake and transport when compared with the LF meal. This can be explained by the higher amount of lipids that are present in the intestine after HF meal consumption, as these lipids need to be taken up and must be transported to other tissue. In agreement, in the intestines of mice, genes related to lipid metabolism were activated when long-term HF intake was compared with LF intake.[28] Also in human muscle biopsies, expression of genes functioning in the lipid metabolism increased after a HF meal.[13,15] In the present study, the expression of genes related to glucose uptake were decreased and those of glucose storage were increased comparing HF with LF consumption, which can be linked to the higher amount of fat in the HF meal and the increased amount of carbohydrates in the LF meal. In line with our results, in human muscle biopsies a shift in glucose metabolism from oxidation to storage was observed, when comparing HF with LF meal consumption.[14] Taken together, these results suggest that both the HF meal and theobromine consumption inhibited glucose metabolism, but through different pathways leading to different physiological effects. Furthermore, the effects of the HF meal on glucose metabolism were more pronounced than those observed after theobromiFinally, after consumption of a HF meal many genes associated with inflammation and immune function were upregulated. A number of studies looking in other tissues and species support our results. In human PBMCs, the consumption of a HF breakfast increased the expression of IL-8 as compared with a LF breakfast.[29] Furthermore, inflammation was one of the most modulated biological processes after HF consumption in the intestines[28] and adipose tissue[30] of mice. In addition, in rat peripheral leukocytes a HF diet increased expression of genes related to leukocyte activation.[31] Our results raise two questions. First, is the effect a primary response to the intake of dietary lipids or a secondary response caused by enterocyte damage? Serum IFABP concentrations, a marker for enterocyte damage,[19] were not different after the LF and HF meals, suggesting that the enterocytes were not damaged after the HF meal. Second, is the acute shift in duodenal pro-inflammatory gene expression profiles translated into an

inflammatory signature in postprandial serum samples? Unfortunately, none of the measured plasma markers showed differences after acute HF and LF consumption. This may indicate that the duodenal inflammatory signal needs longer than a few hours to translate into a systemic inflammatory response, or is not translated at all. Our results on plasma biomarkers are partly contradictory with other studies. Esser et al. found no changes in plasma CRP, ICAM-1, IL-6 and TNF-α, decreases in VCAM-1 and SAA, and an increase in IL-8 after an acute HF breakfast compared with an LF breakfast.[16] Nappo et al. found increased plasma TNF-α, IL6, ICAM-1 and VCAM-1 concentrations comparing HF with LF meal consumption.[32] It should be noted, however, that Esser et al. used a higher amount of fat,[16] while Nappo et al. studied diabetic patients.[32] Also, reported changes in plasma inflammation markers in response to HF and LF feeding have been very variable between studies.[33]

In conclusion, in healthy men, acute theobromine and fat consumption did not change apoA-I expression in the duodenum. Theobromine consumption inhibited intestinal gene expression related to glycogen and glucose breakdown, but did not change those related to lipid and cholesterol metabolism. Furthermore, HF intake activated expression of genes related to lipid and cholesterol uptake and transport and glucose storage, while it decreased those related to glucose uptake. Finally, microarray analyses suggested upregulation of inflammation in the duodenum after HF meal consumption, which was not translated into a systemic inflammatory response directly following a HF meal.

Supplementary data

Figure 1 *P-value distribution after A. adding theobromine (TB) to a low-fat/high-carbohydrate (LF) meal B. comparing high-fat/low-carbohydrate (HF) with LF consumption (n = 8)*

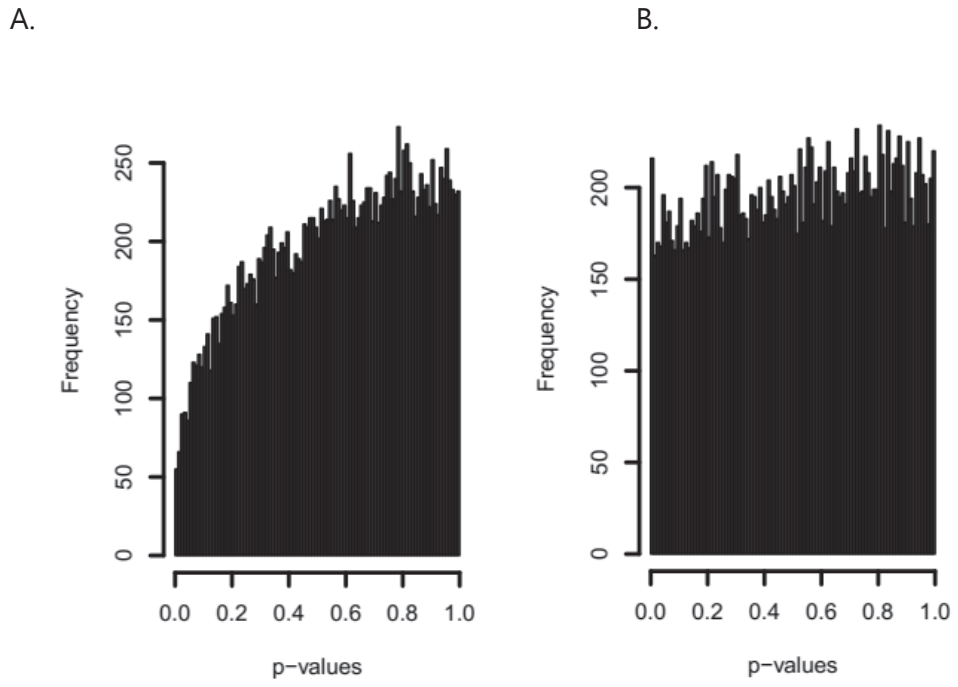


Table 1 *Results of the upstream regulators after adding 850 mg of theobromine (TB) to a low-fat/high-carbohydrate (LF) meal (n = 8)*

Upstream Regulator	Activation z-score	p-value of overlap
U0126	1.71	0.00
streptozocin	1.72	0.00
LY294002	1.99	0.02
PD98059	2.21	0.01
SP600125	2.21	0.05
GATA4	-1.50	0.02
calcitriol	-1.50	0.00
lysophosphatidic acid	-1.59	0.00
PDGF BB	-1.60	0.03
decitabine	-1.67	0.04
AGT	-1.70	0.02
HNF4A	-1.71	0.02
dexamethasone	-1.89	0.00
FADD	-1.94	0.01
Cg	-1.94	0.02
MAP2K1/2	-1.95	0.00
ATP	-1.95	0.01
INS	-1.95	0.04
SOX11	-1.98	0.01
Insulin	-2.08	0.04
aldosterone	-2.17	0.01
EGF	-2.19	0.02
butyric acid	-2.19	0.01
Ca2+	-2.19	0.04
Pkc(s)	-2.19	0.05
GnRH analog	-2.45	0.04
CREB1	-2.88	0.01
Ins1	-3.02	0.00

Table 2 *Results of the GSEA comparing after adding 850 mg of theobromine (TB) to a low-fat/high-carbohydrate (LF) meal (n = 8)*

Gene Set	NES	FDR-q value
KINESINS	2.05	0.154
WP1897.REGULATION.OF.BETA.CELL.DEVELOPMENT	1.96	0.164
WP2739.AMYLOIDS	1.89	0.063
WP1842.KINESINS	1.88	0.068
KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS	1.86	0.089
WP1874.NUCLEOSOME.ASSEMBLY	1.86	0.058
GLUCOSE.METABOLISM	-1.96	0.138
WP716.VITAMIN.A.AND.CAROTENOID.METABOLISM	-1.92	0.142

Table 3 *Top 50 of the results of the upstream regulators after comparing high-fat/low-carbohydrate (HF) with low-fat/high-carbohydrate (LF) consumption (n = 8)*

Upstream Regulator	Activation z-score	p-value of overlap
Bleomycin	2.91	<0.01
IL1B	2.82	<0.01
E. coli B5 lipopolysaccharide	2.79	<0.01
TNFSF11	2.73	<0.01
L-glutamic acid	2.62	0.02
Pirinixic acid	2.57	<0.01
LG100268	2.41	<0.01
5-azacytidine	2.40	0.01
IL1A	2.35	0.01
Linoleic acid	2.31	<0.01
PI3K (complex)	2.31	<0.01
SREBF1	2.27	<0.01
MYD88	2.24	<0.01
Nicotinic acid	2.24	<0.01
CSF1	2.22	<0.01
Kainic acid	2.22	0.01
KLF15	2.20	0.01
IRS1	2.19	<0.01
SCAP	2.18	0.01
Cocaine	2.17	<0.01
AGN194204	2.16	<0.01
Palmitic acid	2.06	<0.01
Jnk	2.04	<0.01
RELA	2.02	0.02

Table 3 *Top 50 of the results of the upstream regulators after comparing high-fat/low-carbohydrate (HF) with low-fat/high-carbohydrate (LF) consumption (n = 8) (continued)*

Upstream Regulator	Activation z-score	p-value of overlap
FABP2	2.00	<0.01
Isoquercitrin	2.00	<0.01
FDFT1	2.00	<0.01
H2AFY	2.00	0.02
IL2RG	2.00	0.05
8-bromoguanosine 3′.5′-cyclic monophosphate	1.99	0.01
Tnf (family)	1.99	0.01
Advanced glycation end-products	1.98	0.02
BCR (complex)	1.98	0.04
CD5	1.98	0.05
PRKCE	1.98	0.04
Gsk3	1.98	0.03
CCL5	1.98	0.04
GCK	-1.98	<0.01
HAND1	-1.98	<0.01
HDL	-1.98	0.02
ARNT	-1.99	<0.01
Dipyridamole	-2.00	<0.01
Calphostin C	-2.00	0.02
Methotrexate	-2.04	<0.01
THRB	-2.20	0.01
15-deoxy-delta-12.14 -PGJ 2	-2.20	0.04
ABCB4	-2.22	<0.01
SB203580	-2.28	<0.01
IL10RA	-2.39	<0.01
GCG	-2.42	<0.01
U0126	-2.48	<0.01
NFE2L2	-2.53	<0.01
Bexarotene	-3.14	0.01

Table 4 *Top 50 of the results of the GSEA after comparing high-fat/low-carbohydrate (HF) with low-fat/high-carbohydrate (LF) consumption (n = 8)*

Gene Set	NES	FDR-q value
KEGG_MALARIA	2.17	0.017
WP143.FATTY.ACID.BETA.OXIDATION	2.09	0.025
CHEMOKINE.RECEPTORS.BIND.CHEMOKINES	2.08	0.020
COLLAGEN.DEGRADATION	2.05	0.022
WP2708.DEGRADATION.OF.COLLAGEN	2.03	0.024
WP2749.METABOLISM.OF.STEROID.HORMONES.AND.VITAMIN.D	1.94	0.057
REGULATION.OF.LIPID.METABOLISM.BY.PEROXISOME.PROLIFERATOR. ACTIVATED.RECEPTOR.ALPHA.PPARALPHA.	1.93	0.057
PPARA.ACTIVATES.GENE.EXPRESSION	1.92	0.058
WP368.MITOCHONDRIAL.LC.FATTY.ACID.BETA.OXIDATION	1.88	0.075
GENERATION.OF.SECOND.MESSENGER.MOLECULES	1.88	0.071
WP2406.CARDIAC.PROGENITOR.DIFFERENTIATION	1.84	0.108
KEGG_CELL.ADHESION.MOLECULES.CAMS.	1.82	0.113
KEGG_CYTOKINE.CYTOKINE.RECEPTOR.INTERACTION	1.82	0.110
WP325.TRIACYLGLYCERIDE.SYNTHESIS	1.82	0.106
WP2797.REGULATION.OF.LIPID.METABOLISM.BY.PEROXISOME. PROLIFERATOR.ACTIVATED.RECEPTOR.ALPHA.PPARALPHA.	1.81	0.104
WP530.CYTOKINES.AND.INFLAMMATORY.RESPONSE	1.80	0.103
KEGG_PPAR.SIGNALING.PATHWAY	1.80	0.104
PPARA_TARGETS	1.80	0.101
KEGG_INTESTINAL.IMMUNE.NETWORK.FOR.IGA.PRODUCTION	1.79	0.106
GLUCOSE.METABOLISM	-2.29	0.001
WP1848.METABOLISM.OF.CARBOHYDRATES	-2.17	0.006
KEGG_PROXIMAL.TUBULE.BICARBONATE.RECLAMATION	-2.14	0.007
RIBOSOMAL.SCANNING.AND.START.CODON.RECOGNITION	-2.05	0.020
KEGG_MINERAL.ABSORPTION	-2.03	0.019
TRANSLATION.INITIATION.COMPLEX.FORMATION	-2.02	0.018
ACTIVATION.OF.THE.MRNA.UPON.BINDING.OF.THE.CAP.BINDING. COMPLEX.AND.EIFS.AND.SUBSEQUENT.BINDING.TO.43S	-1.94	0.045
KEGG_RIBOSOME	-1.94	0.043
WP1889.PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	-1.92	0.045
X3.UTR.MEDIATED.TRANSLATIONAL.REGULATION	-1.88	0.068
GTP.HYDROLYSIS.AND.JOINING.OF.THE.60S.RIBOSOMAL.SUBUNIT	-1.88	0.067
FORMATION.OF.THE.TERNARY.COMPLEX.AND.SUBSEQUENTLY.THE.43S. COMPLEX	-1.86	0.068
L13A.MEDIATED.TRANSLATIONAL.SILENCING.OF.CERULOPLASMIN. EXPRESSION	-1.85	0.072

Table 4 *Top 50 of the results of the GSEA after comparing high-fat/low-carbohydrate (HF) with low-fat/high-carbohydrate (LF) consumption (n = 8) (continued)*

Gene Set	NES	FDR-q value
TRANSLATION	-1.85	0.070
GLUCONEOGENESIS	-1.84	0.074
KEGG_FANCONI.ANEMIA.PATHWAY	-1.83	0.078
WP1828.HEXOSE.TRANSPORT	-1.83	0.073
KEGG_CARBOHYDRATE.DIGESTION.AND.ABSORPTION	-1.83	0.070
PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	-1.82	0.069
FORMATION.OF.A.POOL.OF.FREE.40S.SUBUNITS	-1.82	0.066
WP2683.INFLUENZA.LIFE.CYCLE	-1.82	0.064
KEGG_AMINOACYL.TRNA.BIOSYNTHESIS	-1.82	0.061
MITOCHONDRIAL.TRANSLATION.ELONGATION	-1.82	0.059
EUKARYOTIC.TRANSLATION.ELONGATION	-1.81	0.061
CAP.DEPENDENT.TRANSLATION.INITIATION	-1.81	0.058
BIOC_MTORPATHWAY	-1.81	0.060
EUKARYOTIC.TRANSLATION.INITIATION	-1.81	0.058
MRNA.SPLICING.MAJOR.PATHWAY	-1.79	0.066
WP2773.DEGRADATION.OF.BETA.CATENIN.BY.THE.DESTRUCTION. COMPLEX	-1.78	0.070
MITOCHONDRIAL.TRANSLATION	-1.78	0.068
KEGG_TYROSINE.METABOLISM	-1.78	0.067

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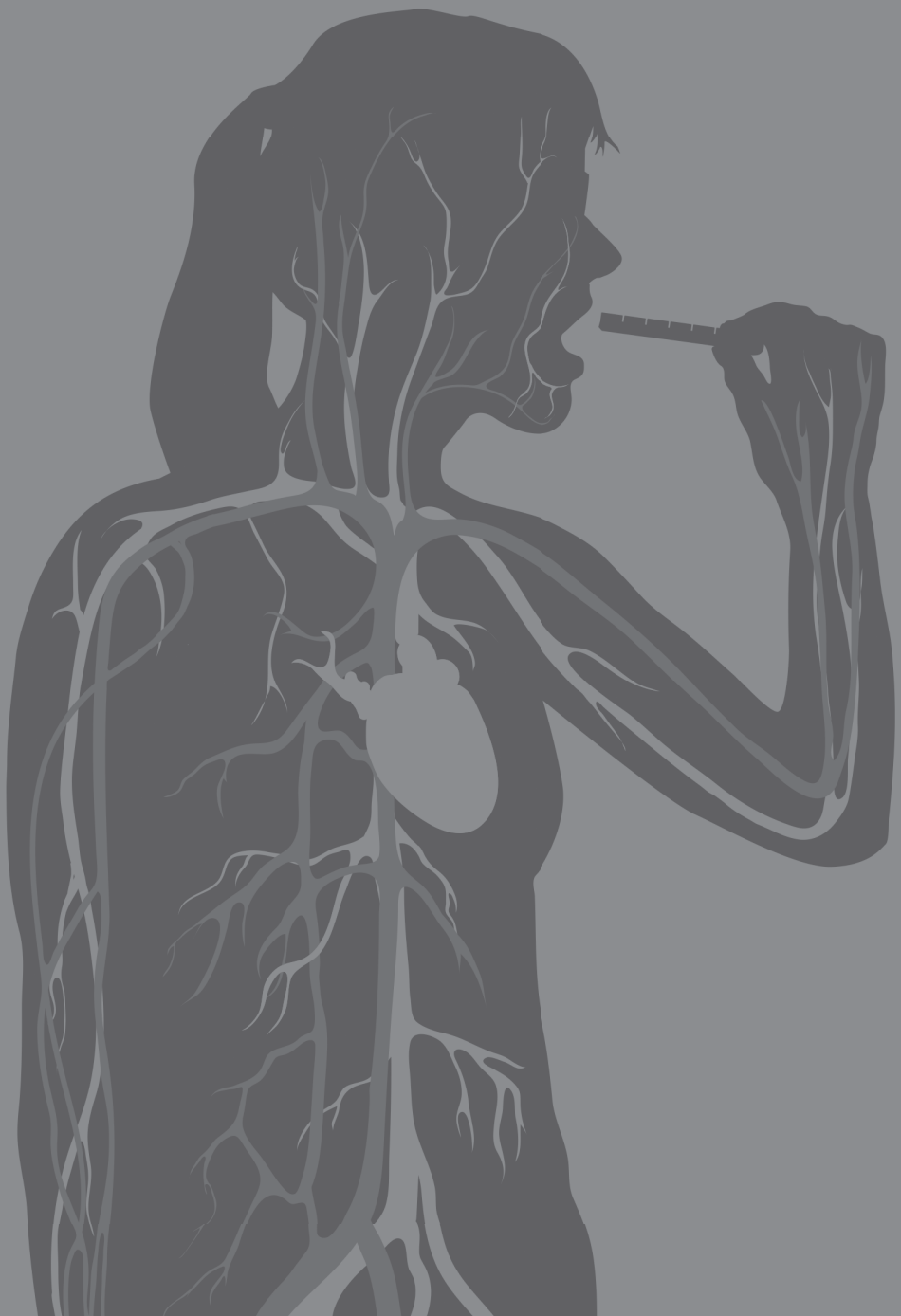

Chapter 5

Theobromine does not affect postprandial lipid metabolism and duodenal gene expression, but has unfavorable effects on postprandial glucose and insulin responses in humans

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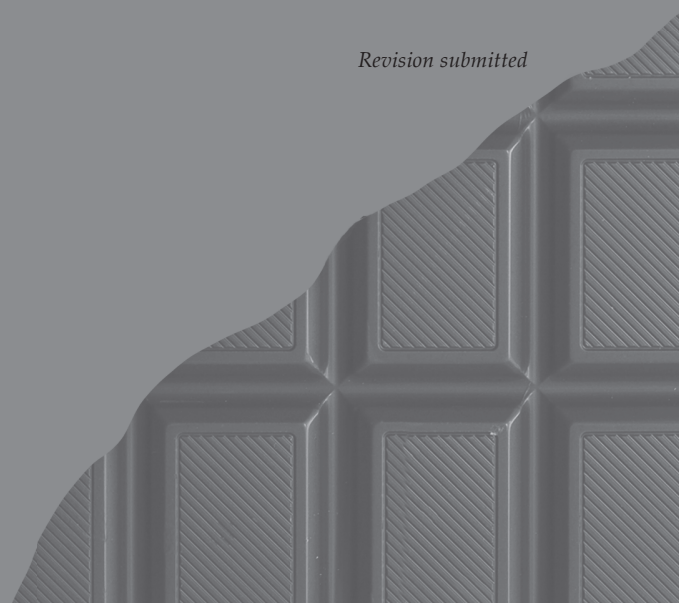
Revision submitted



Abstract

Background & aims Chocolate consumption is associated with a decreased risk for CVD. Theobromine, a compound in cocoa, may explain these effects as it favorably affected fasting serum lipids. However, long-term effects of theobromine on postprandial metabolism as well as underlying mechanisms have never been studied. The objective was to evaluate the effects of 4-week theobromine consumption (500 mg/day) on fasting and postprandial lipid, lipoprotein and glucose metabolism, and duodenal gene expression.

Methods In a randomized, double-blind crossover study, 44 healthy men and women, with low baseline HDL-C concentrations consumed 500 mg theobromine or placebo daily. After 4-weeks, fasting blood was sampled and subjects participated in a 4-hour postprandial test. Blood was sampled frequently for analysis of lipid and glucose metabolism. In a subgroup of 10 men, 5 hours after meal consumption duodenal biopsies were taken for microarray analysis.

Results 4-weeks theobromine consumption lowered fasting LDL-C (-0.21 mmol/L; P = 0.006), and apoB100 (-0.04 g/L; P = 0.022), tended to increase HDL-C (0.03 mmol/L; P = 0.088) and increased hsCRP (1.2 mg/L; P = 0.017) concentrations. Fasting apoA-I, TAG, FFA, glucose and insulin concentrations were unchanged. In the postprandial phase, theobromine consumption increased glucose (P = 0.026), insulin (P = 0.011) and FFA (P = 0.003) concentrations, while lipids and (apo)lipoproteins were unchanged. In duodenal biopsies, microarray analysis showed no consistent changes in expression of genes, pathways or gene sets related to lipid, cholesterol or glucose metabolism.

Conclusions It is not likely that the potential beneficial effects of cocoa on CVD can be ascribed to theobromine. Although theobromine lowers serum LDL-C concentrations, it did not change fasting HDL-C, apoA-I, or postprandial lipid concentrations and duodenal gene expression, and unfavorably affected postprandial glucose and insulin responses. This trial was registered on clinicaltrials.gov under study number NCT02209025.

Introduction

Optimizing dietary intake is a cornerstone for the prevention of many non-communicable diseases such as cardiovascular diseases (CVD), diabetes mellitus type 2, and the metabolic syndrome. In this context, chocolate might have beneficial effects, as high chocolate intake was associated with a 37% reduction in CVD events, a 31% reduction in type II diabetes risk and a 29% reduction in stroke risk.[1] In addition, beneficial effects of cocoa on serum lipid profiles have been demonstrated in many intervention studies. In fact, two different meta-analyses concluded that 2-12 weeks of cocoa consumption significantly decreased low-density lipoprotein cholesterol (LDL-C) and total cholesterol concentrations. However, no effects were found on high-density lipoprotein cholesterol (HDL-C) and triacylglycerol (TAG) concentrations.[2,3] Given the macronutrient composition of chocolate, the potential positive effects of chocolate on serum LDL-C are probably due to one of the minor compounds in cocoa.[4] As dark chocolate contains more cocoa than other chocolate types, dark chocolate should therefore have more favorable metabolic effects than white or milk chocolate. Indeed, Grassi et al. observed that the intake of 100 g of dark chocolate for 15 days increased insulin sensitivity and decreased blood pressure, total cholesterol and LDL-C, while white chocolate did not.[5] Furthermore, Taubert et al. found a decrease in blood pressure, but no changes in plasma lipids or glucose after 18-weeks of dark chocolate consumption compared with white chocolate consumption.[6]

Whether cocoa or dark chocolate also influences postprandial lipid and glucose metabolism has only been explored to a limited extent. This is unfortunate, since evidence is accumulating that disturbances in postprandial lipid and glucose metabolism are important risk markers for CVD.[7,8] In type 2 diabetic patients, Basu et al. (2015) showed increased postprandial HDL-C and insulin concentrations, but no differences in LDL-C, TAG, glucose and high-sensitivity C-reactive protein (hsCRP) concentrations after acute cocoa consumption.[9] In contrast, based on an oral-glucose-tolerance test, insulin sensitivity in healthy subjects improved after 100 g of dark chocolate consumption for 15 days.[10]

An important question is which component in cacao may be responsible for the suggested beneficial fasting and postprandial metabolic effects. Theobromine, a methylxanthine in cocoa, is a promising candidate [11] given its beneficial effects on blood pressure [10] and fasting plasma lipids.[12] So far, effects of theobromine on postprandial metabolism have not been examined. Therefore, the aim of the present study was to evaluate the effects of 4-weeks pure theobromine intake (500 mg/day) on fasting and postprandial lipid, lipoprotein and glucose metabolism. We were especially interested in changes in HDL metabolism, since theobromine has been reported to increase fasting apolipoprotein A-I (apoA-I) concentrations,[12] which may

decrease CVD risk.[13] Therefore, overweight and slightly obese subjects with low HDL-C concentrations were included, as these subjects may be more responsive to interventions targeting HDL metabolism. Potential underlying mechanisms were addressed by performing microarray analyses in duodenal biopsies.

Material and methods

Study population

Apparently healthy middle-aged and elderly overweight and slightly obese men and women (BMI 25-35 kg/m^2) were recruited in University and hospital buildings by posters, in local newspapers via advertisements, and among participants who had participated in earlier studies from our Department. They were invited for two screening visits with an interval of ≥1 week. During the screening visits body weight without heavy clothing, height, and blood pressure were determined. Blood pressure was measured in fourfold using an Omron M7 (Omron Healthycare Europe B.V., Hoofddorp, The Netherlands). The first measurement was not used and the final three measurements were averaged. Furthermore, a fasting blood sample was taken for analysis of serum total cholesterol, HDL-C, and plasma glucose concentrations. In addition, subjects had to complete a general and medical questionnaire. Inclusion criteria were: men aged between 45-70 years, and women aged between 50-70 years to exclude pregnant women, since theobromine can cross the placenta,[14] BMI between 25 and 35 kg/m^2, fasting serum HDL-C concentrations <1.2 mmol/L for men and <1.5 mmol/L for women so as to include participants with HDL-C concentrations below the 50th percentile of the Dutch population,[15] fasting serum total cholesterol concentrations <8.0 mmol/L, fasting plasma glucose concentrations <7.0 mmol/L, stable body weight (weight gain or loss <3 kg in the previous 3 months), no use of lipid-lowering, anti-diabetic or anti-hypertensive medication or a medically prescribed diet, no history or current gastrointestinal diseases or complaints, no use of vitamin or fish oil supplements, no diabetes, no abuse of alcohol or drugs, no smoking, and no active or history of coronary artery disease. In addition, subjects had not participated in another biomedical study for the past 30 days. After information about the aim of the study was given and the potential risks of the experimental procedures were discussed, all participants gave their written informed consent before entering the study. Forty-eight participants were included. After inclusion, subjects were urged not to change their dietary habits, levels of physical exercise, and alcohol intake during the study. The study was performed according to the guidelines laid down in the Declaration of Helsinki. The protocol was approved by the Medical

Ethical Committee of the University Hospital Maastricht and the trial was registered on clinicaltrials.gov under study number NCT02209025.

Study design and product

The study had a randomized, double blind, placebo-controlled, crossover design and consisted of 2 intervention periods of 4-weeks separated by a 4-week washout period. From 2-weeks before the start of the study and during the study, participants were instructed by a research dietician to avoid products containing cocoa, for which they received a detailed list with products. Furthermore, the consumption of caffeine-containing drinks was restricted to a maximum of 4 cups a day, since theobromine is a metabolite of caffeine. In theory, these 4 caffeine-containing drinks could result in the formation of maximally 80 mg of theobromine,[16] which was ±16% of the daily experimental theobromine intake (500 mg) as provided by us. During the test days, caffeine intake was prohibited. Based on a computer-generated randomization scheme, subjects were allocated to a group starting with theobromine or placebo drinks. At breakfast, subjects consumed daily drinks (20 ml) enriched with 500 mg theobromine or placebo. The experimental and placebo drinks were matched for composition, appearance and taste (Supplementary data, Table 1). Theobromine was obtained from Fagron (Uitgeest, the Netherlands) and drinks were produced by Pharmavize (Mariakerke, Belgium). The drinks were provided in boxes of eight 20 ml flasks and participants received 2 boxes at the start of the 4-week intervention period and 2 boxes halfway the intervention period. The drinks and boxes were color-coded to blind the participants and investigators. Subjects were required to return all empty bottles and unused drinks, which were counted to estimate compliance. At the end of the two intervention periods, subjects had to record their habitual food intake of the previous 4 weeks by completing a food frequency questionnaire (FFQ). From these FFQs, energy and nutrient intakes were calculated using the Dutch Nutrient databank (NEVO 2014). FFQs were immediately checked by the research dietician in the presence of the subjects. Participants recorded in diaries any signs of illness, medication used, alcohol consumption, and any deviations from the study protocol and other complaints.

Visits, postprandial test, test meal and biopsies

All subjects visited the University at the start of the study (day 1), and twice in the fourth week (days 25 and 28) of both the experimental and placebo periods to measure blood pressure, heart rate and body weight, and to take a fasting blood sample (no food or drinks, except water, 12 hours before the visit). All visits were in the morning and volunteers arrived at the metabolic research unit of our Department by public transport or car to standardize measurements as much as possible. Furthermore,

subjects were not allowed to use drinks containing alcohol and strenuous activities 48 hours before the visits.

At day 28 of both experimental periods, subjects participated in a postprandial test. To minimize differences in dietary intake before these test days, subjects were provided with a standard dinner the evening before the test day, which consisted of a commercially available lasagne (638 kcal, 28.4 g protein, 44.0 g carbohydrates and 37.6 g fat). At the start of the postprandial test day, blood pressure and heart rate were measured, an intravenous cannula was inserted in a forearm vein and a fasting blood sample was collected (T0). Next, subjects were asked to consume a high-fat mixed meal and their test drink within 10 minutes. This meal provided 965 kcal, 17.9 g proteins, 85.7 g carbohydrates and 60.6 g fat (Table 1).

Table 1 *Composition of the test meal*

Nutrient	Amount
Energy (kcal)	965
Protein (g)	17.9
Energy (%)	7
Carbohydrates (g)	85.7
Energy (%)	35
Mono- and disaccharides (g)	45.6
Polysaccharides (g)	40.1
Total fat (g)	60.6
Energy (%)	56
Saturated fatty acids (g)	36.0
Monounsaturated fatty acids (g)	18.7
Polyunsaturated fatty acids (g)	4.1
Cholesterol (mg)	341

After meal intake, the volunteers were not allowed to eat or drink anything, except water. Postprandial blood samples were taken at T = 15 (T15), T = 30 (T30), T = 45 (T45), T = 60 (T60), T = 90 (T90), T = 120 (T120) and T = 240 (T240) minutes after meal consumption. A subgroup of 10 men was willing to participate in an additional duodenoscopy to sample duodenal biopsies. Additional inclusion criteria for this supplementary measurement were willingness to undergo duodenoscopy, for which also informed consent was obtained. Five hours after meal intake, duodenal biopsies were taken at the Department of Endoscopy. During duodenoscopy, no sedatives were given. Four duodenal mucosal tissue samples, just proximal of the ampulla of Vater, were taken using standard biopsy forceps. The diameter of the biopsies varied between 2.0 mm and 2.2 mm. After sampling, biopsies were immediately frozen

in liquid nitrogen, stored at -80˚C and at the end of the study analyzed for gene expression profiles.

Blood sampling

Fasting blood was sampled in serum, EDTA- and NaF-vacutainer tubes from a forearm vein by the same person in the same room, and generally at the same time of the day. During the postprandial test, serum and NaF-vacutainer tubes were sampled at each time point. Furthermore, at T0, T60, T120 and T240, blood was sampled in EDTA-vacutainer tubes. Directly after sampling, the EDTA- and NaF-tubes were put on ice and centrifuged at 1300 x g for 15 min at 4˚C within 60 min to obtain plasma. Serum tubes were allowed to clot for 1 hour at 20˚C, and then centrifuged at 1300 x g for 15 min at 20˚C to obtain serum. Serum and plasma aliquots were stored at -80˚C until analyses. At the end of the study, samples from one subject were analyzed within the same analytical run.

Analysis

In all fasting serum samples, concentrations of total cholesterol (CHOD-PAP method; Roche Diagnostics System, Mannheim, Germany), HDL-C (precipitation method; Roche Diagnostics System, Mannheim, Germany), TAG (Trigl; Roche, Mannheim, Germany), insulin (human insulin-specific radioimmunoassay (RIA) kit, Linco Research, Missouri, USA), apoA-I, apolipoprotein B100 (apoB100) (Horiba ABX, Montpellier Cedex, France) and hsCRP (Horiba ABX, Montpellier, France) were measured. In all fasting NaF plasma samples, glucose (Roche Diagnostic Systems, Woerden, the Netherlands) and free fatty acid (FFA) concentrations were measured (Wako Biochemicals, Richmond, USA). Theobromine, caffeine and paraxanthine concentrations were measured in fasting serum samples of day 28, as described.[17] Liver and kidney function parameters (creatinin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total bilirubin, and gamma-glutamyltransferase (gamma-GT)) were measured in fasting serum samples from day 28 (Beckman Coulter Synchron LX20 PRO Clinical System, Beckman Coulter Inc., Fullerton, CA, USA).

Fasting LDL-C was calculated using the Friedewald formula.[18] As this formula is not accurate when fasting serum TAG concentrations exceeds 4.52 mmol/L, LDL-C could not be calculated for 3 subjects. The homeostasis model assessment for insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI) were calculated to estimate the degree of insulin resistance.[19, 20]

In all postprandial serum samples HDL-C, TAG, apoA-I and insulin concentrations and in all postprandial NaF-samples glucose and FFA concentrations were measured. Serum apolipoprotein B48 (apoB48) concentrations (Shibayagi Gunma, Japan) were measured at T0, T30, T60, T90, T120 and T240.

Microarray processing and data analysis

Total RNA was extracted from a frozen mucosal duodenal sample using TRIzol reagent (Invitrogen, Breda, the Netherlands) with purification on columns using Qiagen RNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Total RNA (100 ng) was labeled by Whole Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19697 unique genes (Affymetrix, Santa Clara, CA). Microarray analyses were performed as described.[21] In short, microarrays were normalized with the robust multichip average method and probes were annotated as described.[22,23] This gene set was filtered on an expression of >20 on at least 5 arrays and measured with ≥5 probes. The filtered data set consisted of 12294 genes. Individual genes were defined as changed when comparison of the normalized signal intensities showed a $p \leq 0.05$ in a 2-tailed paired intensity-based moderated t-statistics and a fold change of >1.2 or <-1.2.[24] Further functional data analysis was performed on the filtered dataset with ingenuity pathway analysis (IPA), upstream regulators and Gene Set Enrichment Analysis (GSEA).[25] Pathways were selected on –log p-values of <1.3, which indicates a significant change of $p<0.05$ in that specific pathway. In the upstream regulator analysis, significant linked gene sets were selected using a p-value of <0.05 for gene expression and a p-value of overlap of <0.05. A z-score above 1.5 indicates activation, whereas a z-score below -1.5 indicates inhibition of this upstream regulator. In the GSEA, gene sets were selected on a False Discovery Rate (FDR) q-value of <0.2 and were ranked on the Normalized Enrichment Score (NES).

Statistical analysis

If available, fasting concentrations of days 25 and 28 were averaged. Parameters were checked for normality using the Shapiro-Wilk test. Fasting parameters were tested for carryover effect, which were absent.[26] Effects of theobromine consumption on fasting parameters, that were normally distributed, were evaluated using the mixed models procedure with subject as random factor, and period and treatment as fixed factors, and reported as estimated marginal means ± SDs. Fasting TAG concentrations, which were not normally distributed, were log transformed and reported as geometric means with 95% CI. Differences in hsCRP concentrations and liver and kidney function parameters were not normally distributed, also not after log transformation, and were therefore evaluated by a Wilcoxon signed-rank test, and reported as medians with ranges. Since not all FFQ results were normally distributed, also not after log transformation, energy and nutrient intakes were evaluated by a Wilcoxon signed-rank test, and reported as medians with ranges. Relationships between changes in theobromine, caffeine and paraxanthine concentrations with those in lipid and lipoproteins were evaluated with the Pearson's correlation coefficient. Spearman's rank correlation coefficients were calculated for changes in hsCRP

and changes in theobromine, caffeine, paraxanthine, glucose, lipid and lipoprotein concentrations.

Postprandial responses were analyzed by linear mixed models with subject as between subject variable, treatment and time as fixed factors and a treatment*time interaction. If this interaction term did not reach statistical significance, it was omitted from the model. If the factor time was significant, time points were compared to baseline concentrations, using Bonferroni's corrections for multiple comparisons. For TAG, apoB48, FFA, glucose and insulin, the incremental area under the curves (iAUC) or the decremental area under the curves (dAUC) were calculated using the trapezoidal rule.[27] Since only the iAUC for apoB48 was normally distributed, and log transformation did not result in normality for all parameters, the iAUCs, maximal increases and time to peak values are reported as medians with ranges, and effects of theobromine were tested using a Wilcoxon signed-rank test. The iAUCs were tested for carryover effect. None of the parameters showed significance.[26]

Differences between subject characteristics of the total group (n = 21 men) and the subgroup (n = 10 men) in which duodenal biopsies were sampled, were tested with an independent t-test. Results were considered to be statistically significant if $p \leq 0.05$. All statistical analyses were performed using SPSS 20.0 for Mac (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics and compliance

A flow diagram of the participants throughout the study is presented in Figure 1. After screening, 48 subjects were eligible for participation and started the study. During the first intervention period, four participants discontinued participation. At that time, three subjects (2 men and 1 woman) received the theobromine drinks. One man withdrew, because of non-study related illness and the woman because she did not like the taste of the drinks. The other man was excluded in the second week of the study, because of non-compliance, i.e. he had consumed less than 80% of the drinks provided. Finally, one woman receiving the placebo drinks had to stop, because she was diagnosed with CVD by her cardiologist. This appointment was already planned before the start of the study without informing the project team. Baseline characteristics of the 44 participants who completed the study are shown in Table 2.

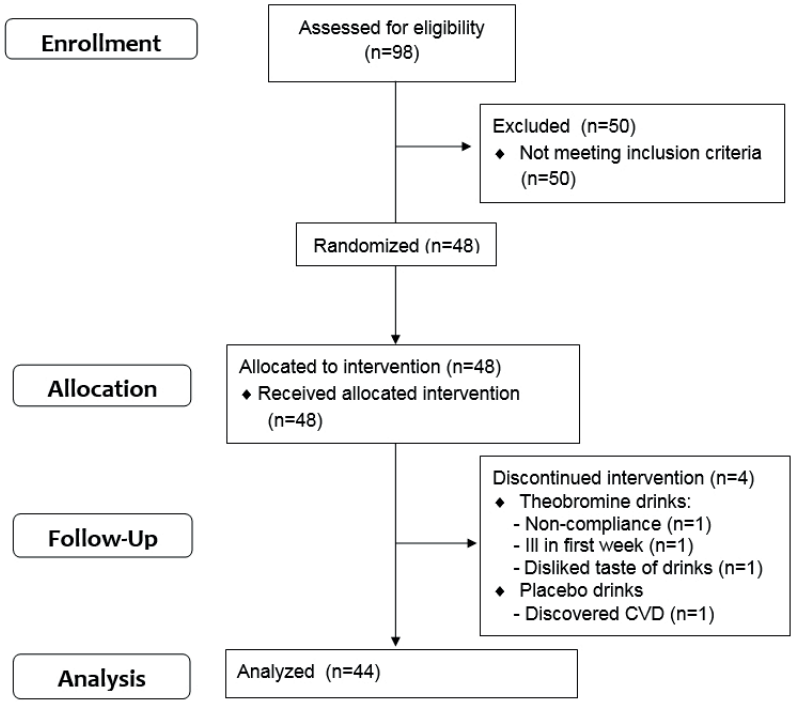


Figure 1 *Flow chart of participant inclusion throughout the study*

Based on the number of returned bottles, mean compliance was 99% (range: 89 - 100%) during both intervention periods. To further substantiate compliance, serum theobromine concentrations were measured. Fasting serum theobromine concentrations significantly increased after the theobromine period as compared with placebo period (placebo 0.6 ± 0.6 μmol/L vs. theobromine 7.0 ± 6.6 μmol/L, $P < 0.001$) (Figure 2). Forty-three of the 44 participants had higher theobromine concentrations after the theobromine period than during the placebo period. Furthermore, also plasma caffeine and paraxanthine concentrations were significantly increased after theobromine consumption (caffeine: placebo, 5.4 ± 5.1 μmol/L vs. theobromine, 6.9 ± 6.0 μmol/L, $P = 0.013$ and paraxanthine: placebo, 1.5 ± 1.2 μmol/L vs. theobromine, 1.9 ± 1.4 μmol/L, $P = 0.022$). Changes in serum theobromine concentrations did not correlate with changes in serum caffeine and paraxanthine concentrations, but changes in serum caffeine concentrations correlated positively with changes in serum paraxanthine concentrations ($r = 0.740$, $P < 0.001$).

Table 2 *Baseline characteristics of the participants who completed the study*

	Mean ± SD
Age (years)	60.3 ± 5.5
BMI (kg/m^2)	29.2 ± 3.0
Total cholesterol (mmol/L)	5.65 ± 0.92
HDL-C (mmol/L)	1.22 ± 0.18
Glucose (mmol/L)	5.56 ± 0.63
SBP (mmHg)	134 ± 15
DBP (mmHg)	86 ± 9
Heart rate (bpm)	70 ± 12

[1] *Values are mean ± SD. n = 44. bpm: beats per min, DBP: diastolic blood pressure, HDL-C: high density lipoprotein cholesterol, SBP: systolic blood pressure.*

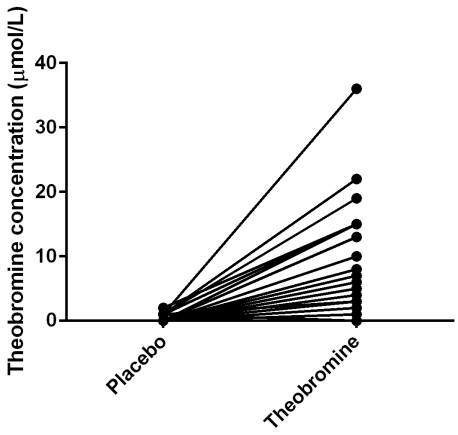


Figure 2 *Individual theobromine concentrations after 4-weeks of placebo or theobromine consumption (n = 44).*

FFQ

Energy, protein, carbohydrate, fat, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, alcohol, cholesterol or fiber intakes between the 2 periods were comparable (Supplementary data, Table 2).

Fasting lipid, lipoprotein and glucose metabolism

Fasting serum LDL-C (-0.21 mmol/L; 95% CI: -0.35, -0.06; P = 0.006), apoB100 (-0.04 g/L; 95% CI: -0.07, -0.01; P = 0.022) and total cholesterol (-0.10 mmol/L; 95% CI: -0.28, -0.02; P = 0.029) concentrations were significantly reduced at the end of the theobromine period as compared with the placebo period. Fasting serum HDL-C tended to increase after theobromine consumption (0.03 mmol/L; 95% CI: 0.00, 0.06; P = 0.088), while apoA-I concentrations were comparable between the 2 periods (0.01

g/L; 95% CI: -0.02, 0.04; P = 0.576). As a consequence, the ratios of total cholesterol/HDL-C (-0.23; 95% CI: -0.39, -0.07; P = 0.006) and LDL-C/HDL-C (-0.29; 95% CI: -0.44, -0.15; P < 0.001) were significantly lower after theobromine consumption. Finally, fasting serum TAG, FFA, glucose, and insulin concentrations, as well as HOMA-IR, QUICKI, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were not significantly changed after theobromine consumption (Table 3).

Table 3 *Fasting lipids, (apo)lipoproteins, glucose, insulin, and metabolic risk markers after 4-weeks of theobromine consumption*[1]

	Placebo	Theobromine	Difference
LDL-C (mmol/L)[2]	3.75 ± 0.91	3.54 ± 0.75	-0.21 ± 0.45*
ApoB100 (g/L)	1.24 ± 0.26	1.21 ± 0.24	-0.04 ± 0.13*
Total cholesterol (mmol/L)	5.71 ± 1.10	5.56 ± 1.01	-0.10 ± 4.44*
HDL-C (mmol/L)	1.09 ± 0.19	1.11 ± 0.21	0.03 ± 0.13
ApoA-I (g/L)	1.39 ± 0.16	1.40 ± 0.18	0.01 ± 0.13
Total cholesterol / HDL-C	5.41 ± 1.38	5.18 ± 1.36	-0.23 ± 0.53*
LDL-C / HDL-C	3.53 ± 1.01	3.24 ± 0.89	-0.29 ± 0.46*
TAG (mmol/L)[3]	0.31 (0.47 - 0.57)	0.44 (0.52 - 0.62)	0.06 (-0.01 - 0.13)
FFA (μmol/L)	408 ± 147	385 ± 118	23 ± 126*
Glucose (mmol/L)	6.01 ± 0.62	6.02 ± 0.61	0.01 ± 0.27
Insulin (μU/mL)	15.78 ± 5.42	15.50 ± 6.54	-0.28 ± 3.45
HOMA-IR	2.09 ± 0.72	2.07 ± 0.86	-0.02 ± 0.46
QUICKI	0.31 ± 0.02	0.32 ± 0.02	0.00 ± 0.01
BMI (kg/m^2)	29.2 ± 3.0	29.2 ± 3.1	0.0 ± 0.40
SBP (mmHg)	134 ± 14	135 ± 14	0 ± 7
DBP (mmHg)	86 ± 9	86 ± 10	1 ± 7
Heart rate (bpm)	62 ± 9	63 ± 9	1 ± 7

[1] *Values are estimated marginal means ± SD. n = 44. * Significantly different from placebo (linear mixed models): * p<0.05. apoA-I: apolipoprotein A-I, apoB100: apolipoprotein B100, bpm: beats per min, DBP: diastolic blood pressure, FFA: free fatty acids, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, SBP: systolic blood pressure, TAG: triacylglycerol.*

[2] *n = 41 because of missing values*

[3] *Values are geometric mean with 95% CI in parentheses*

Postprandial lipid, lipoprotein and glucose metabolism

No significant treatment*time interactions were found during the postprandial period. However, time effects were significant for all parameters (P < 0.001; Figure 3). Theobromine intake had no effects on postprandial changes in HDL-C, apoA-I, TAG and apoB48, but did affect those of FFA, glucose and insulin (Figure 3, Table 5). After theobromine consumption, the decrease in postprandial FFA concentrations was less pronounced (P = 0.002), the iAUC was increased (P = 0.003) and the maximal FFA concentrations were increased (P < 0.001). Further, theobromine consumption increased the postprandial glucose response (P = 0.001) as well as the iAUC (P =

0.026). Postprandial insulin concentrations tended to increase after theobromine consumption (P = 0.052), while the iAUC was increased (P = 0.011). Furthermore, maximal postprandial insulin concentrations were increased (P = 0.005; Figure 3, Table 5).

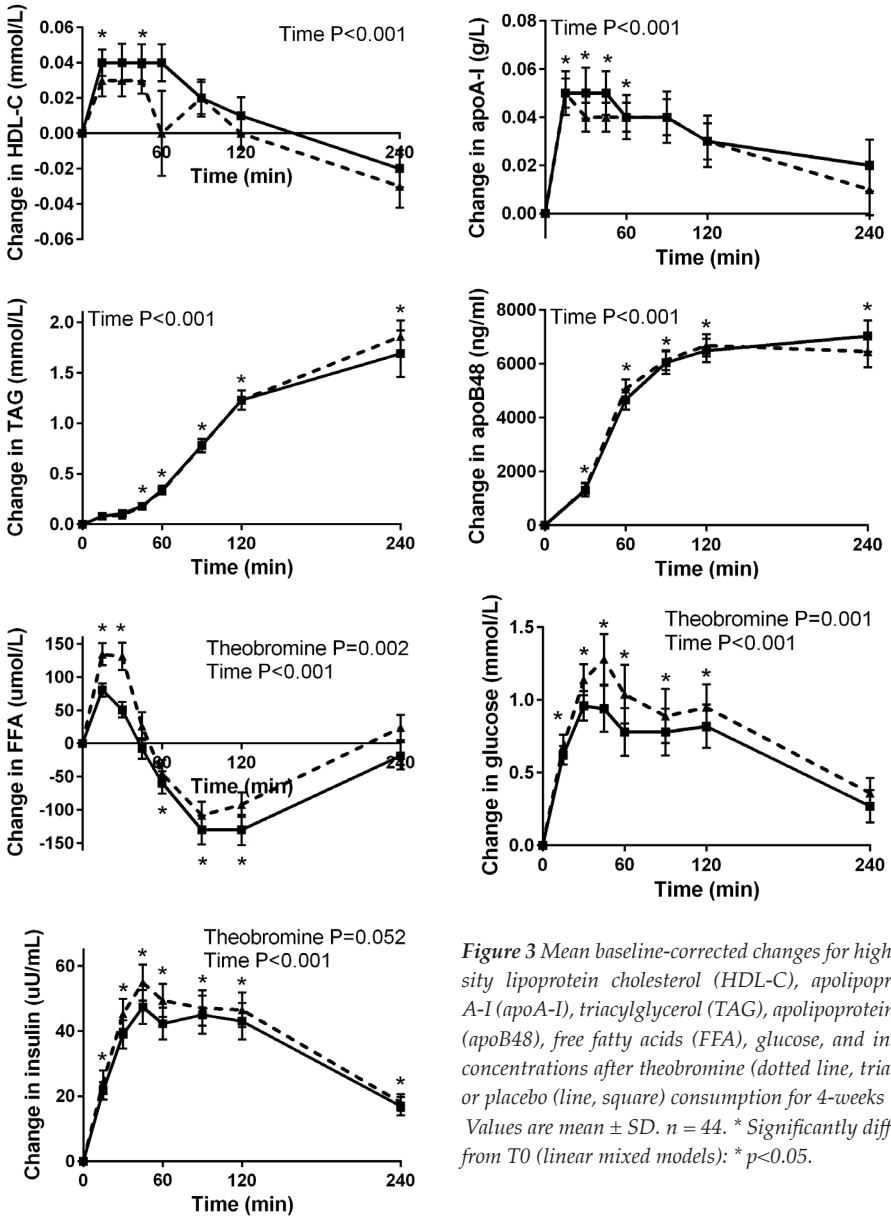


Figure 3 *Mean baseline-corrected changes for high density lipoprotein cholesterol (HDL-C), apolipoprotein A-I (apoA-I), triacylglycerol (TAG), apolipoprotein B48 (apoB48), free fatty acids (FFA), glucose, and insulin concentrations after theobromine (dotted line, triangle) or placebo (line, square) consumption for 4-weeks*
*Values are mean ± SD. n = 44. * Significantly different from T0 (linear mixed models): * p<0.05.*

Table 5 *iAUC, dAUC, maximal increase and time to peak or dip for triacylglycerol (TAG), apolipoprotein B48 (apoB48), free fatty acids (FFA), glucose and insulin after 4-weeks theobromine consumption*[1]

		iAUC/dAUC [2]		Maximal peak in/decrease [3]		Time to peak/dip (min)	
		Placebo	**Theobromine**	**Placebo**	**Theobromine**	**Placebo**	**Theobromine**
TAG	iAUC	249 (7 - 608)	219 (58 - 518)	1.9 (0.1 - 5.1)	1.8 (0.6 - 5.2)	240 (120 - 240)	240 (120 - 240)
ApoB48	iAUC	11.1 (2.7 - 23.0)	12.3 (1.4 - 24.1)	7.0 (2.2 - 18.4)	6.7 (1.4 - 16.4)	180 (60 - 240)	120 (60 - 240)
FFA	iAUC	3.3 (0.0 - 19.6)	6.9 (0.0 - 38.7) *	117 (0 - 279)	154 (0 - 534) *	23 (0 - 240)	30 (0 - 240)
FFA	dAUC	12.1 (92.7 - 0.1)	11.2 (60.9 - 0.0)	97 (618 - 0)	108 (349 - 0)	90 (60 - 240)	90 (45 - 240)
Glucose	iAUC	126 (3 - 689)	151 (6 - 726) *	1.4 (0.3 - 4.0)	1.5 (0.4 - 4.1)	45 (15 - 240)	45 (15 - 240)
Insulin	iAUC	7.1 (1.0 - 39.6)	7.3 (0.0 - 20.7) *	53.0 (14.8 - 247.5)	64.1 (0.0 - 192.7) *	60 (15 - 240)	60 (0 - 240)

[1] *Values are medians with ranges. n = 44. * Significantly different from placebo (Wilcoxon signed-rank test): * $p<0.05$.*

[2] *TAG (mmol*min/L), apoB48 (ng*10^5*min/mL), FFA (mol*10^4*min/L), glucose (mmol*min/L) and insulin (U*10^3*min/mL)*

[3] *TAG (mmol/L), apoB48 (mg/mL), FFA (mol/L), glucose (mmol/L) and insulin (U/mL)*

Hs-CRP and markers reflecting liver and kidney function

Theobromine had no effect on parameters related to liver and kidney function. In addition, values remained for all subjects within reference ranges during the entire study (Table 4). However, theobromine consumption increased plasma hsCRP concentration with 1.2 mg/L (P = 0.017, Table 4). As elevated hsCRP concentrations are associated with changes in fasting lipid, (apo)lipoprotein[28] and glucose concentrations,[29] correlations with changes in hsCRP were calculated. Only significant correlations with changes in TAG (r = -0.332, P = 0.028) and apoA-I (r = -0.383, P = 0.010) were found. Changes in hsCRP concentrations did not correlate with those in theobromine (r = 0.041, P = 0.790).

Table 4 *Markers reflecting liver and kidney function and hsCRP after 4-weeks theobromine consumption*[1]

	Placebo	Theobromine	Reference values[2] m / f	
Creatinine (μmol/L)	86 (60 – 112)	85 (58 – 104)	60-115	50-100
GammaGT (U/L)	27 (13-88)	25 (13 - 70)	<55	<38
ASAT (U/L)	28 (17 – 42)	27 (18 – 63)	<35	<31
ALAT (U/L)	24 (16 – 50)	24 (12 – 47)	<45	<34
Bilirubin (μmol/L)	8.7 (4.3 – 33.8)	8.1 (3.7 – 31.7)	<20	<20
hsCRP (mg/L)	1.7 (0.2 - 14.5)*	2.8 (0.1 - 5.0) *		

[1] *Values are medians with ranges. n = 44. * Significantly different from placebo (Wilcoxon signed-rank test): * p<0.05. ALAT: alanine aminotransferase, ASAT: aspartate aminotransferase, gamma-GT: gamma-glutamyl-transferase, hsCRP: high sensitivity c-reactive protein.*

[2] *Reference values are specific for the clinical chemistry laboratory of the Academic Hospital Maastricht*

Gene expression and pathway analysis

Baseline characteristics and responses to theobromine consumption of the men (n = 10), in which duodenal biopsies were taken, were comparable to those of the other men (n = 21). Only baseline glucose concentrations were significantly higher (6.07 ± 0.78 mmol/L vs. 5.34 ± 0.42 mmol/L) in the subgroup in which duodenal biopsies were taken (data not shown).

Theobromine significantly changed the expression of only 100 genes (Figure 4). None of these genes were related to lipid, cholesterol or glucose metabolism. IPA showed that 9 pathways were changed (Table 6), which were again not related to lipid, cholesterol or glucose metabolism. GSEA revealed that 3 gene sets were downregulated, whereas 364 gene sets were upregulated after theobromine consumption (Supplementary data, Table 3). Many of these gene sets were related to the cell cycle or the immune system. Also the 53 upstream regulators that were changed were not related to lipid, cholesterol or glucose metabolism (Supplementary data, Table 4).

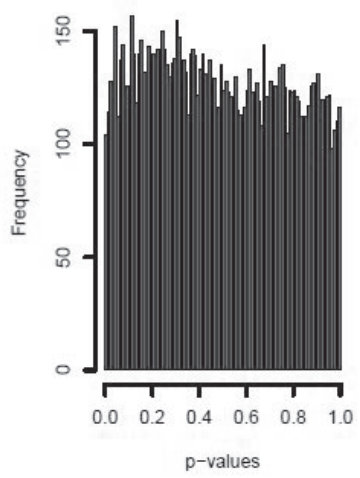


Figure 4 *P-value distribution of the microarray analysis of duodenal biopsies after 4-weeks of theobromine consumption (n = 10)*

Table 6 *IPA results of the microarray analysis of duodenal biopsies after 4-weeks of theobromine consumption*

Pathway	-log(p-value)	Ratio
Mitotic Roles of Polo-Like Kinase	1.30	0.06
Eicosanoid Signaling	1.34	0.06
CDP-diacylglycerol Biosynthesis I	1.36	0.13
Sulfate Activation for Sulfonation	1.38	0.50
Glycine Biosynthesis I	1.38	0.50
Glutamate Biosynthesis II	1.38	0.50
Glutamate Degradation X	1.38	0.50
Cell Cycle: G2/M DNA Damage checkpoint Regulation	1.72	0.08
p38 MAPK Signaling	1.93	0.06

[1] *n = 10.*

Discussion

This randomized, double-blind, placebo-controlled human intervention study showed that a daily intake of 500 mg theobromine for 4-weeks decreased fasting serum total cholesterol, LDL-C, and apoB100 concentrations, tended to increase fasting serum HDL-C concentrations, and significantly increased those of hsCRP. During the postprandial phase, theobromine consumption increased both glucose and insulin responses, which suggests a lower insulin sensitivity. In addition, theobromine increased postprandial FFA concentrations, while TAG, HDL-C, apoA-I and apoB48 responses did not change. These findings suggest that it is highly unlikely that theobromine is the component in cocoa that explains the epidemiological findings suggesting that high dark chocolate consumption reduces CVD risk.

Theobromine has been reported to increase fasting serum HDL-C and apoA-I concentrations.[12] However, in the present study, fasting serum HDL-C concentrations only tended to increase, while apoA-I concentrations were unchanged. With 44 participants, the statistical power to detect a difference of 0.07 g/L in serum apoA-I concentrations, as reported by Neufingerl et al., was >95% ($\alpha = 0.05$), when a within-subject variability of 0.10 g/L was used. There are at least three explanations for these contradicting findings. First, Neufingerl et al. used 850 mg theobromine, while the intake in our study was 500 mg. Second, Neufingerl and colleagues added the theobromine to a milk-based drink, while we dissolved the theobromine in water.[12] It is possible that theobromine needs one or more components from milk to effectively increase serum apoA-I and HDL-C concentrations. Third, Neufingerl et al. studied healthy subjects, while we included overweight or obese participants with relatively low HDL-C concentrations. Further, in our study serum HDL-C concentrations tended to increase while apoA-I concentrations did not, which may suggest that HDL-C particle composition - and possibly HDL functionality - changed. In agreement with the study of Neufingerl et al. (2013), serum LDL-C and apoB100 concentrations also decreased.[12]

So far, no other study has examined the effects of theobromine on fasting or postprandial glucose metabolism. For cocoa, beneficial effects on fasting insulin concentrations and insulin resistance has been reported.[30] However, effects on postprandial glucose metabolism are inconsistent. One study found increased postprandial insulin responses, but no differences in those of glucose after acute cocoa consumption,[9] while another study reported lower glucose and insulin responses after dark chocolate consumption for 15 days.[10] In our study, theobromine unfavorably affected postprandial glucose and insulin responses. We can only speculate about the mechanism underlying these responses. It has been reported that theobromine inhibits cyclic adenosinemonophosphate (cAMP)-phosphodiesterase,[31,32] which increases

cellular cAMP levels, leading to a net increase in hepatic glucose production.[33] This may result in higher plasma glucose concentrations and consequent higher insulin responses. In addition, cAMP amplifies the exocytosis of insulin granules in the pancreas,[34] which may also have contributed to the observed higher circulating postprandial insulin concentrations. Another mechanism could relate to the hepatic metabolism of theobromine, where cytochrome P450 2E1 (CYP2E1) and cytochrome P450 1A2 (CYP1A2) are involved.[35] It is known that the activity of CYP2E1 can be upregulated by its own substrates.[36] Therefore, higher theobromine intakes may increase CYP2E1 activity, which causes oxidative stress, leading to hepatic insulin resistance.[37] In fact, CYP2E1 knock out mice are protected against insulin resistance, obesity and hyperlipidemia.[38] Finally, hsCRP concentrations increased after theobromine consumption, which is also associated with insulin resistance.[39] hsCRP is an acute phase protein, which can be produced in the liver in response to factors (e.g. interleukin 6 (IL-6)) released by adipocytes and macrophages.[40] Whether this pathway was upregulated by theobromine intake warrants further study.

Theobromine had no effects on postprandial lipid and lipoprotein metabolism. FFA concentrations normally decrease following meal consumption.[41,42] However, we found here that in both intervention periods, FFA concentrations slightly increased during the first 45 min after meal consumption preceding the expected decrease. This increase was more pronounced and the decreases less pronounced after theobromine consumption, for which we have no explanation.

Microarray analysis showed that only few genes in duodenal biopsies were differentially expressed and did not suggest regulation of pathways, gene sets or upstream regulators related to lipid or glucose metabolism after 4-weeks of theobromine consumption. We therefore conclude that theobromine does not change duodenal gene expression of genes related to lipid and glucose metabolism. The changes observed in this study are thus caused in other organs.

Compliance was excellent as evidenced by the increased serum theobromine concentrations. Furthermore, also the concentrations of serum caffeine and paraxanthine, a metabolite of caffeine, increased after theobromine consumption. The hepatic enzymes - CYP1A2 and CYP2E1 - that metabolize theobromine also function in caffeine metabolism.[41] Therefore, it is possible that these enzymes are occupied by theobromine and have a limited capacity to metabolize caffeine, leading to higher circulating caffeine concentrations. In the liver, caffeine is mainly metabolized into paraxanthine by CYP1A2.[42] Theoretically, higher caffeine concentrations may therefore also lead to higher paraxanthine concentrations. Consequently, it is also possible that the effects on lipid and glucose metabolism observed in this study are caused by the increases in caffeine and/or paraxanthine concentrations and not only by the elevation of theobromine.

This study has several limitations. First, we studied a relatively homogenous group in terms of ethnicity, age, BMI, and HDL-C concentrations and it remains to be determined to what extent these findings can be extrapolated to other population groups. Furthermore, participants consumed the theobromine at breakfast and the fasting blood samples were taken 24 hours later. Peak plasma concentrations of theobromine are usually seen 3 hours after theobromine consumption and have returned to baseline 24 hours after intake.[43] Therefore, it can be speculated that effects of theobromine on fasting metabolism have already disappeared 24 hours after intake. However, as also no effects on postprandial lipids were observed, this speculation is not very likely.

In conclusion, it is not likely that the potential beneficial effects of cocoa on CVD can be ascribed to theobromine. Although theobromine improves serum LDL-C concentrations, it did not change fasting HDL-C, apoA-I, or postprandial lipid concentrations and duodenal gene expression, and unfavorably affected postprandial glucose and insulin responses.

Supplementary data

Table 1 *Composition of the test drinks (20mL)*

	Theobromine drink	Placebo drink
Theobromine (mg)	500	-
Microcrystalline cellulose (mg)	-	500
Methyl cellulose (mg)	150	150
Sucralose (mg)	10	10
Sodium benzoate (mg)	100	100
Anise 0.1% (mg)	20	20
Water	Till 20 g	Till 20 g

Table 2 *Habitual dietary intake during the study*[1]

	Placebo	Theobromine
Energy (kcal/day)	2315 (1014 - 3673)	2312 (887 - 3571)
Protein (E%)	15.9 (10.2 - 23.9)	15.2 (11.2 - 21.6)
Carbohydrates (E%)	42.7 (33.6 - 56.9)	42.1 (34.0 - 54.6)
Total fat (E%)	37.4 (23.0 - 47.8)	37.3 (25.7 - 48.4)
Saturated fatty acids (E%)	12.1 (6.6 - 17.2)	12.2 (6.2 - 15.7)
Monounsaturated fatty acids (E%)	12.8 (8.4 - 23.1)	12.9 (9.1 - 20.5)
Polyunsaturated fatty acids (E%)	7.9 (4.4 - 11.7)	7.9 (3.7 - 12.9)
Alcohol (E%)	1.4 (0.0 - 13.3)	1.3 (0.0 - 10.0)
Cholesterol (mg/day)	240 (89 - 469)	228 (97 - 429)
Fiber (g/day)	27.5 (16.4 - 48.0)	26.2 (14.0 - 44.2)

[1] Values are medians with ranges. $n = 44$.

Table 3 *Results of the GSEA, including all upregulated gene sets and the top 50 of the downregulated gene sets, of the duodenal biopsies after 4-week of theobromine consumption*[1]

Gene Set	NES	FDR-q value
KEGG_MINERAL.ABSORPTION	2.01	0.12
WP1871.NEUROTRANSMITTER.RELEASE.CYCLE	1.99	0.076
SYNTHESIS.OF.LEUKOTRIENES.LT.AND.EOXINS.EX.	1.93	0.105
CHROMOSOME.MAINTENANCE	-2.93	<0.001
WP2652.MITOTIC.PROMETAPHASE	-2.90	<0.001
MITOTIC.PROMETAPHASE	-2.87	<0.001
MITOTIC.M.M.G1.PHASES	-2.83	<0.001
RESOLUTION.OF.SISTER.CHROMATID.COHESION	-2.80	<0.001
CELL.CYCLE.MITOTIC	-2.77	<0.001
M.PHASE	-2.75	<0.001
TELOMERE.MAINTENANCE	-2.73	<0.001
MITOTIC.ANAPHASE	-2.67	<0.001
WP2446.RB.IN.CANCER	-2.67	<0.001
MITOTIC.METAPHASE.AND.ANAPHASE	-2.65	<0.001
SEPARATION.OF.SISTER.CHROMATIDS	-2.65	<0.001
NUCLEOSOME.ASSEMBLY	-2.62	<0.001
WP2757.MITOTIC.METAPHASE.AND.ANAPHASE	-2.59	<0.001
DEPOSITION.OF.NEW.CENPA.CONTAINING.NUCLEOSOMES. AT.THE.CENTROMERE	-2.59	<0.001
WP466.DNA.REPLICATION	-2.57	<0.001
WP1874.NUCLEOSOME.ASSEMBLY	-2.55	<0.001
WP1928.TELOMERE.MAINTENANCE	-2.55	<0.001
KEGG_DNA.REPLICATION	-2.50	<0.001
KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS	-2.44	<0.001
MITOTIC.PROPHASE	-2.44	<0.001
MEIOSIS	-2.38	<0.001
WP1925.SYNTHESIS.OF.DNA	-2.38	<0.001
EXTENSION.OF.TELOMERES	-2.38	<0.001
WP1775.CELL.CYCLE.CHECKPOINTS	-2.37	<0.001
KEGG_CELL.CYCLE	-2.37	<0.001
CONDENSATION.OF.PROPHASE.CHROMOSOMES	-2.37	<0.001
MITOTIC.G1.G1.S.PHASES	-2.35	<0.001
DNA.STRAND.ELONGATION	-2.34	<0.001
DNA.REPLICATION	-2.34	<0.001
DNA.REPLICATION.PRE.INITIATION	-2.34	<0.001
WP2739.AMYLOIDS	-2.34	<0.001
CELL.CYCLE.CHECKPOINTS	-2.34	<0.001
ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX	-2.33	<0.001
WP1858.MITOTIC.G1.G1.S.PHASES	-2.32	<0.001
DNA.METHYLATION	-2.32	<0.001
G1.S.TRANSITION	-2.31	<0.001
WP179.CELL.CYCLE	-2.31	<0.001
MEIOTIC.RECOMBINATION	-2.30	<0.001

Table 3 *Results of the GSEA, including all upregulated gene sets and the top 50 of the downregulated gene sets, of the duodenal biopsies after 4-week of theobromine consumption[1] (continued)*

Gene Set	NES	FDR-q value
MEIOTIC.SYNAPSIS	-2.29	<0.001
WP2785.M.G1.TRANSITION	-2.28	<0.001
WP2772.S.PHASE	-2.27	<0.001
M.G1.TRANSITION	-2.26	<0.001
WP1782.APC.C.MEDIATED.DEGRADATION.OF.CELL.CYCLE. PROTEINS	-2.26	<0.001
DNA.DAMAGE.TELOMERE.STRESS.INDUCED.SENESCENCE	-2.26	<0.001
WP619.TYPE.II.INTERFERON.SIGNALING.IFNG.	-2.26	<0.001
PACKAGING.OF.TELOMERE.ENDS	-2.25	<0.001
G2.M.CHECKPOINTS	-2.24	<0.001
PRC2.METHYLATES.HISTONES.AND.DNA	-2.24	<0.001
APC.C.CDC20.MEDIATED.DEGRADATION.OF.MITOTIC. PROTEINS	-2.24	<0.001

[1] $n = 10$.

Table 4 *Results of the upstream regulator analysis of duodenal biopsies after 4-week of theobromine consumption[1]*

Upstream Regulator	Activation z-score	p-value of overlap
CSF2	-4.21	<0.001
ESR1	-4.01	<0.001
PTGER2	-4.00	<0.001
HGF	-3.47	<0.001
Vegf	-3.46	<0.001
TAL1	-3.43	<0.001
RABL6	-3.32	<0.001
TBX2	-3.30	<0.001
FOXM1	-2.90	<0.001
CCND1	-2.54	<0.001
ERBB2	-2.43	<0.001
MYC	-2.30	0.030
TSC2	-2.24	0.042
estrogen	-2.20	0.024
E2F1	-2.19	<0.001
E2F3	-2.13	<0.001
beta-estradiol	-2.05	0.004
S100A6	-2.00	0.002
SMOC2	-2.00	<0.001
EP400	-1.98	0.008
RARA	-1.89	<0.001
E2f	-1.56	<0.001
4-hydroxytamoxifen	1.50	0.024
valproic acid	1.67	0.016
COL18A1	1.67	0.008

Table 4 *Results of the upstream regulator analysis of duodenal biopsies after 4-week of theobromine consumption[1] (continued)*

Upstream Regulator	Activation z-score	p-value of overlap
KDM5B	1.88	<0.001
SPARC	1.89	0.006
miR-145-5p	1.95	0.019
RBL1	1.96	<0.001
Rb	1.98	0.003
PPRC1	2.00	0.012
carbonyl cyanide m-chlorophenyl hydrazone	2.00	0.019
PAX6	2.00	0.023
BMS-690514	2.00	0.039
PGR	2.05	0.020
tretinoin	2.10	0.006
doxorubicin	2.11	0.008
bee venom	2.24	0.013
fluocinolone acetonide	2.24	0.003
RBL2	2.24	<0.001
RB1	2.27	0.001
LY294002	2.28	0.005
triamcinolone acetonide	2.38	0.024
CDKN1A	2.41	<0.001
BNIP3L	2.45	<0.001
TCF3	2.53	<0.001
mir-21	3.05	0.013
Irgm1	3.15	<0.001
CDKN2A	3.55	0.001
TP53	3.80	<0.001
let-7	3.92	<0.001
calcitriol	3.94	<0.001
NUPR1	4.35	<0.001

[1] $n = 10$.

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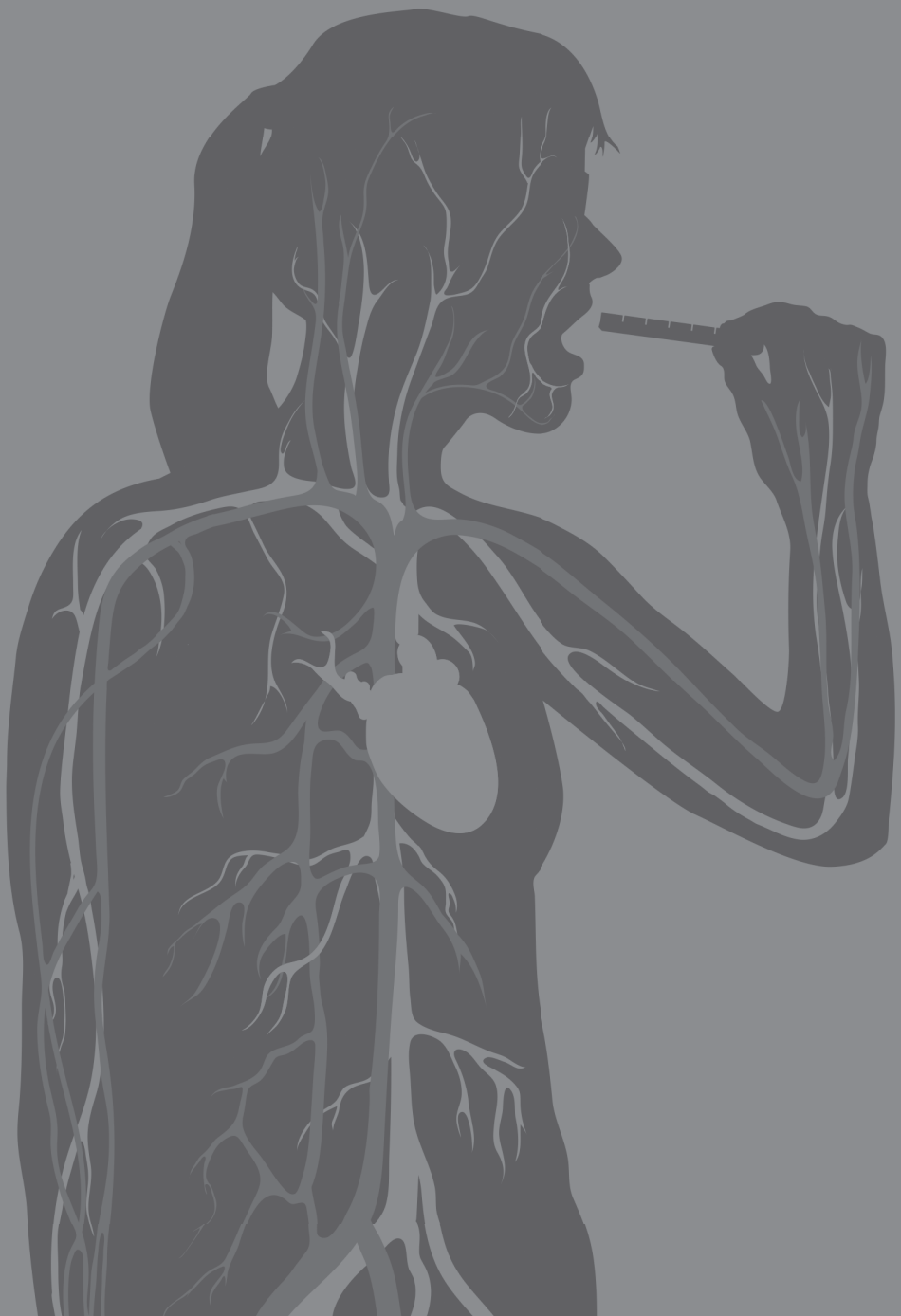
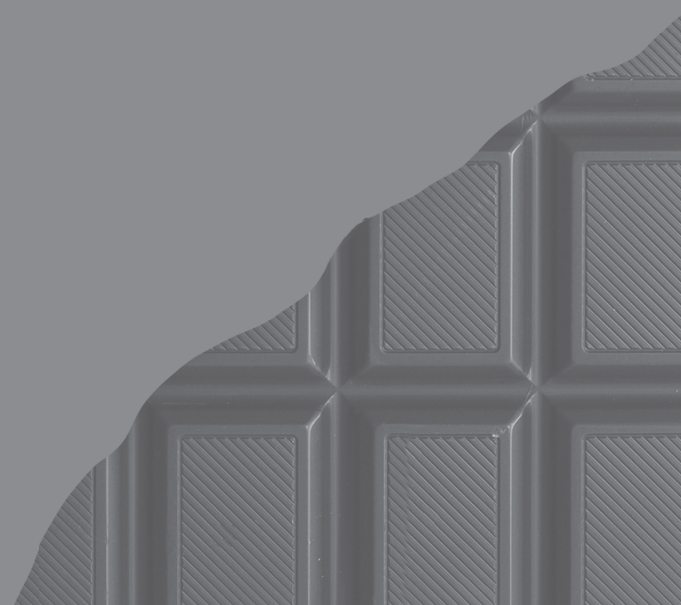


Chapter 6

Theobromine consumption does not improve fasting and postprandial vascular function in overweight subjects

Lotte Smolders, Ronald P. Mensink, Jogchum Plat

To be submitted



Abstract

Introduction Theobromine, a component of cocoa, may favorably affect conventional lipid-related cardiovascular risk markers, but effects on flow-mediated dilation (FMD) and other vascular function markers are not known.

Objective To evaluate the effects of 4-weeks theobromine consumption (500 mg/day) on fasting and postprandial vascular function markers.

Design In a randomized, double-blind crossover study, 44 apparently healthy overweight men and women with low HDL-C concentrations, consumed daily 500 mg theobromine or placebo for 4-weeks. After 4-weeks, FMD, peripheral arterial tonometry (PAT), augmentation index (AIx), pulse wave velocity (PWV), blood pressure (BP) and retinal microvasculature measurements were performed. These measurements were carried out under fasting conditions and 2.5-hours after a mixed meal challenge.

Results 4-weeks theobromine consumption did not change fasting vascular function markers, except for a decrease in central AIx (cAIx, -1.7 pp, $P = 0.037$) and a trend towards smaller venular calibers (-2 μm, $P = 0.074$). Meal consumption decreased FMD (0.89 pp, $P = 0.002$), reactive hyperemia index (RHI, -0.30, $P < 0.001$), peripheral systolic BP (SBP, -3 mmHg, $P \leq 0.001$), peripheral diastolic BP (DBP, -2 mmHg, $P \leq 0.001$), central SBP (-6 mmHg, $P \leq 0.001$) and central DBP (-2 mmHg, $P \leq 0.001$), but increased heart rate (HR, 2 bmp, $P < 0.001$). Theobromine did not modify these postprandial effects, but increased postprandially the brachial artery diameter (0.03 cm, $P = 0.015$), and decreased the cAIx corrected for a HR of 75 (cAIx75, -5.0 pp, $P = 0.004$) and peripheral AIx (pAIx, -6.3 pp, $P = 0.017$).

Conclusion Theobromine consumption did not improve fasting and postprandial endothelial function, but increased postprandial peripheral arterial diameters and decreased the AIx. These findings do not suggest that theobromine alone contributes to the proposed cardioprotective effects of cocoa. This trial was registered on clinicaltrials.gov under study number NCT02209025.

Introduction

Chocolate consumption is associated with a lower risk for cardiovascular diseases (CVD).[1] These effects are specifically evident for dark chocolate, which has been shown to improve serum lipid profiles,[2,3] to reduce blood pressure,[2,4] to increase insulin sensitivity, and to improve vascular endothelial function as measured with flow-mediated dilation (FMD).[2] The components from cocoa responsible for the potentially beneficial effects on FMD are unknown, but it can be argued that theobromine contributes to these effects. In fact, the acute consumption of low amounts of theobromine (111 mg) and caffeine (11 mg) together with cocoa flavanols increased the postprandial FMD.[5] Furthermore, a 4-week study showed that theobromine consumption lowered fasting low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (apoB) concentrations,[6,7] and increased those of serum high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (apoA-I). Recently, however, we could not confirm these latter findings.[7] Moreover, we observed no effects of theobromine consumption on postprandial lipid responses, while postprandial free fatty acid, glucose and insulin responses were increased.[7] It is known that both postprandial hyperlipedemia[8] and hyperglycemia[9] impair vascular function. Therefore, it is relevant to examine effects of theobromine, not only on fasting vascular function, but also on vascular resilience after a meal challenge.

FMD is a non-invasive vascular function marker to assess endothelial function and an accepted predictive biomarker for future CVD events.[10] Another method to evaluate endothelial function is peripheral arterial tonometry (PAT), which measures the reactive hyperaemia index (RHI) of the small arteries and is negatively correlated with the presence of CVD risk factors.[11] Furthermore, several non-invasive markers exist to assess arterial stiffness such as carotid-femoral pulse wave velocity (PWVcf) and the augmentation index (AIx). PWVcf is associated with a higher frequency of stroke, CVD and total mortality,[12] while the AIx is associated with higher CVD risk.[13] Finally, the microvasculature can be studied by evaluating the caliber of the blood vessels in the retina. Cross-sectional wider venules and narrower arterioles predict an increased risk of CVD events in women, but not in men.[14]

In agreement with effects on FMD,[2] dark chocolate consumption also improved RHI.[15] However, effects on measures of arterial stiffness are conflicting. In one study, no effects were found on PWVcf,[13] while in another study beneficial effects were found on AIx.[16,17] Furthermore, Terai et al. showed no differences in arteriolar and venular width after short-term dark chocolate consumption.[12] So far, long-term effects of theobromine consumption on a wide panel of vascular function markers have never been studied. Therefore, we examined the effects of 4-weeks theobromine

consumption (500 mg/day) on FMD, RHI, pulse wave velocity (PWV), AIx, and retinal microvasculature in fasting conditions and after a meal challenge.

Material and methods

Study population

Details of this study have been published before.[7] Briefly, 48 healthy overweight or slightly obese (BMI 25-35 kg/m^2) men (45-70 years) and women (50-70 years) participated. During two screening visits, with an interval of ≥1-week, blood pressure was measured in fourfold using an Omron M7 (Omron Healthycare Europe B.V., Hoofddorp, the Netherlands). The first measurement was discarded and the last three measurements were averaged. Furthermore, a fasting blood sample was taken for analysis of serum total cholesterol, HDL-C, and plasma glucose concentrations. Inclusion criteria were: fasting serum HDL-C concentrations below the 50th percentile of the Dutch population (<1.2 mmol/L for men and <1.5 mmol/L for women),[18] fasting serum total cholesterol concentrations <8.0 mmol/L, fasting plasma glucose concentrations <7.0 mmol/L, and no use of lipid-lowering, anti-diabetic or anti-hypertensive medication or a medically prescribed diet. All participants gave their written informed consent before entering the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. The study protocol was approved by the Medical Ethical Committee of the University Hospital Maastricht. The trail was registered at clinicaltrials.gov under study number NCT02209025.

Study design and product

This study with a randomized, double-blind, cross-over design consisted of 2 intervention periods of 4-weeks separated by a 4-week washout period. Starting 2-weeks before the first intervention period and during the entire study, participants were instructed by a research dietician to avoid cocoa-containing products, for which they received a detailed list with products. Since theobromine is a metabolite of caffeine, the consumption of caffeine-containing drinks was restricted to a maximum of 4 cups a day and volunteers were instructed not to change their intake throughout the study. Subjects consumed in random order a drink (20ml) enriched with theobromine (500mg/day) or placebo every day during breakfast (Supplementary data, Table 1). Theobromine was obtained from Fagron (Uitgeest, the Netherlands) and drinks were produced and provided by Pharmavize (Mariakerke, Belgium).

Test day and test meal

At the end of the 4-week intervention and placebo periods, subjects visited the University in fasting condition (no food or drinks, except water, 12-hours before the visit). To minimize effects of the previous meal, we provided all subjects with the same commercially available lasagne (638 kcal, 28.4 g protein, 44.0 g carbohydrates and 37.6 g fat) the evening before each of the two test days. Furthermore, subjects were asked to avoid alcohol consumptions and strenuous activities 48-hours before a visit.

In the morning, volunteers arrived at the Department by public transport or car, to standardize measurements as much as possible. Upon arrival and after a 10 min rest in supine position, vascular function measurements were performed in fasting conditions. Next, subjects were asked to consume a high-fat mixed meal (965 kcal, 17.9 g protein, 86.7 g carbohydrates, 60.6 g fat and 341 mg cholesterol) together with their experimental drink, within 10 min. For the next 2.5-hours following the meal, participants were not allowed to eat or drink anything except water. After 2.5-hours (T150), the same panel of vascular function measurements was performed in the same order, using the same protocols.

Vascular measurements

Investigators were blinded during the study and data analyses. All vascular measurements were performed in a quiet and temperature controlled (22°C) room. Peripheral systolic blood pressure (pSBP), peripheral diastolic blood pressure (pDBP), FMD, RHI, PWVcf, AIx and retinal vascular image measurements were determined as described before.[19] Furthermore, carotid-radial PWV (PWVcr) was measured with the SphygmoCor, as described for the PWVcf.[19] Central systolic blood pressure (cSBP) and central diastolic blood pressure (cDBP) values were obtained from the SphygmoCor measurements.

Statistical analysis

Before the start of the study, it was calculated that the statistical power to detect a true difference of at least 1.20 percent points (pp) in FMD between the experimental and control period was over 80% (α=0.05), when 43 subjects successfully completed the study. For these calculations, a within-subject variability of 2.82 pp in FMD[20] was used. As the expected drop-out rate was 10%, the aim was to recruit 48 men and women.

All data is presented as mean ± SD unless indicated otherwise. All parameters were checked for normal distributions with the Shapiro-Wilk test. Fasting measurements after 4-weeks of placebo or theobromine intervention were compared with the general mixed model procedure with subject as random factor, and treatment

and period as fixed factors. Differences in postprandial changes after 4-weeks of placebo or theobromine interventions were also evaluated with general mixed models with subject as random factor and treatment and meal as fixed factors and a treatment*meal interaction. If this treatment*meal interaction was not significant, it was omitted from the model. Results were considered statistically significant if $p \leq 0.05$. All statistical analyses were performed using SPSS 20.0 for Mac (SPSS Inc., Chicago, IL, USA).

Results

Study participants

After screening, 48 subjects were eligible for participation and started the study. During the first intervention period, 4 participants (1 male and 3 female) discontinued the study. Thus, 44 participants completed the study. The flow diagram and subject characteristic are presented as Supplementary data (Figure 1 and Table 2). RHI data was missing for three persons, due to technical problems. For 2 subjects (1 man, 1 woman), T0 values were missing and for 1 male participant, a T150 value was absent. For 4 persons CRAE and CRVE calibers were missing (1 man and 3 women) and for 2 persons (1 man and 1 woman) the T150 values were missing, because of a poor quality of the fundus photos.

Fasting vascular function

In the fasting condition, theobromine consumption did not change FMD, brachial artery diameter, and RHI. Furthermore, PWVcf, PWVcr, pAIx and cAIx75 did not change, but the cAIx was significantly lower after theobromine intake (-1.7 pp, 95% CI: -6.1, -0.2, P = 0.037). The CRAE and AVR also remained stable during the study, but theobromine intake tended to decrease the CRVE (-2 μm, 95% CI: -4, 0, P = 0.074). Finally, fasting BP and HR were not affected (Table 1).

Postprandial vascular function

As expected, test meal intake significantly decreased FMD (-0.89 pp, 95% CI: -1.43, -0.35, P = 0.002) and RHI (-0.30, 95% CI: -0.43, -0.16, P < 0.001) responses, but these effects did not depend on theobromine consumption. However, the brachial artery diameter increased when theobromine was part of the test meal (0.03 cm, 95% CI: 1.23, 4.45, P = 0.015 for treatment*meal effects).

Table 1 *Brachial diameter, brachial artery FMD, RHI, PWVcf, PWVcr, cAIx, cAIx75, pAIx, CRAE, CRVE, AVR, pSBP, pDBP, cSBP, cDBP and HR in fasting (T0) and postprandial (T150) condition after 4-weeks of placebo or theobromine consumption*[1]

	Placebo			Theobromine		
	T0	**T150**	**Change**	**T0**	**T150**	**Change**
Brachial diameter (cm)	0.49 ± 0.06	0.50 ± 0.08	0.00 ± 0.04	0.49 ± 0.07	0.52 ± 0.08	0.03 ± 0.04*
Brachial artery FMD (%)[$]	4.87 ± 2.54	3.87 ± 2.32	-1.00 ± 2.97	4.43 ± 2.01	3.65 ± 2.25	-0.78 ± 2.48
RHI[2,$]	2.64 ± 0.68	2.38 ± 0.61	-0.24 ± 0.65	2.58 ± 0.61	2.23 ± 0.47	-0.35 ± 0.60
PWVcr (m/s)	7.1 ± 1.1	7.1 ± 1.1	-0.1 ± 1.2	7.4 ± 1.3	7.1 ± 1.5	-0.3 ± 1.6
PWVcf (m/s)	9.0 ± 1.4	9.0 ± 1.6	0.0 ± 1.3	8.8 ± 1.6	9.0 ± 1.5	0.2 ± 1.5
cAIx (%)	28.3 ± 9.9	21.9 ± 10.5	-6.4 ± 6.2	26.6 ± 10.4[#]	15.2 ± 11.2	-11.3 ± 8.4
pAIx (%)	-14.8 ± 14.9	-24.1 ± 13.7	-9.3 ± 10.6	-16.8 ± 15.3	-32.4 ± 13.8	-15.6 ± 14.4*
cAIx75 (%)	21.6 ± 8.7	16.3 ± 9.6	-5.3 ± 6.5	21.4 ± 9.3	11.2 ± 10.7	-10.3 ± 8.2*
CRAE (μm)[3]	135 ± 19	135 ± 19	0 ± 9	134 ± 19	136 ± 19	2 ± 6
CRVE (μm)[3]	230 ± 14	231 ± 13	0 ± 5	228 ± 14	231 ± 13	2 ± 7
AVR[3]	0.59 ± 0.09	0.59 ± 0.09	0.00 ± 0.05	0.58 ± 0.09	0.59 ± 0.09	0.01 ± 0.04
pSBP (mmHg)[$]	134 ± 14	132 ± 12	-3 ± 9	134 ± 14	130 ± 13	-4 ± 10
pDBP (mmHg)[$]	85 ± 10	83 ± 8	-2 ± 5	86 ± 10	83 ± 9	-3 ± 6
cSBP (mmHg)[$]	126 ± 13	121 ± 11	-5 ± 8	125 ± 12	118 ± 13	-7 ± 10
cDBP (mmHg)[$]	86 ± 9	84 ± 9	-2 ± 6	87 ± 9	84 ± 9	-3 ± 5
HR (bpm)[$]	62 ± 9	64 ± 10	2 ± 4	62 ± 8	65 ± 10	3 ± 7

[1] *Values are mean ± SD. n = 44.*

Linear mixed models were conducted to find significant differences. [#] *p<0.05 for fasting differences from placebo,* * *p<0.05 for treatment*meal effects,* [$] *P<0.05 for meal effects.*

[2] *n=42 at T0, n=41 at T150 due to missing values,*

[3] *n=41 at T0, n=39 at T150 due to missing values.*

Abbreviations: FMD: flow mediated dilation, RHI: reactive hyperemia index, PWV: pulse wave velocity, PWVcf: PWV of the carotis-femoralis, PWVcr: PWV of the carotis-radialis, cAIx: central augmentation index, cAIx75: cAIx corrected for a heart rate of 75, pAIx: peripheral augmentation index, CRAE: mean arteriolar width, CRVE: mean venular width, AVR: arteriolar to venular ratio, p: peripheral, c: central, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate.

Test meal consumption did not affect arterial stiffness as measured via PWVcf and PWVcr. These effects were not changed when theobromine was added to the test meal. However, theobromine as part of the test meal tended to decrease the postprandial cAIx (-4.9 pp, 95% CI: -5.8, -0.7, P = 0.080 for treatment*meal effects) and decreased the pAIx (-6.3 pp, 95% CI: -9.2, -2.4, P = 0.017 for treatment*meal effects) and cAIx75 (-5.0 pp, 95% CI: -6.8, -2.4, P = 0.004 for treatment*meal effects). Test meal consumption did not change retinal vascular calibers. Effects were not changed by theobromine consumption. Finally, the test meal significantly decreased cSBP (-5 mmHg, 95% CI: -8, -4, $p \leq 0.001$), cDBP (-2 mmHg, 95% CI: -3, -1, $p \leq 0.001$), pSBP (-3 mmHg, 95% CI: -5, -1, $p \leq 0.001$) and pDBP (-2 mmHg, 95% CI: -3, -1, $p \leq 0.001$) and increased HR (2 bpm, 95% CI: 1, 3, $p \leq 0.001$). These effects were not modified by theobromine (Table 1).

Discussion

This randomized, double-blind, placebo-controlled intervention study showed that a daily intake of 500 mg theobromine for 4-weeks did not affect FMD, RHI, PWV and the retinal microvasculature in fasting and postprandial conditions. However, theobromine consumption increased brachial arterial diameters and decreased the AIx during the postprandial phase.

The amount of 500 mg theobromine provided corresponds to approximately 67-100 g of dark chocolate.[21] It has been shown that consumption of 100 g dark chocolate for 15 days increased fasting FMD by 1.5 pp,[22] which has been explained by an increase in nitric oxide (NO) concentrations due to a higher endothelial-derived NO synthase activity.[23] Our study was adequately powered to detect such a change. Moreover, the finding that fasting RHI - which is also NO-mediated but more related to the small arteries and the microvasculature - did not change after theobromine consumption was also opposite to the effects observed after consuming cocoa.[15] Furthermore, theobromine did not modify the effects of a meal challenge on vascular resilience. It is well known that a meal high in fat or high in carbohydrates impairs endothelial function.[24,25] During postprandial hyperlipemia and hyperglycemia, the production of reactive oxygen species increases, which decreases NO bioavailability and thereby endothelial function.[24,25] Indeed, also our test meal stressed the endothelium, as evidenced by decreases in postprandial FMD and RHI values, which is in agreement with other studies.[26-28] In contrast to our results, flavanol-rich cocoa consumption ameliorated the decrease in FMD after intake of a fatty meal,[29] while flavonoid-rich dark chocolate consumption even increased FMD values one hour after intake.[30] Our data, therefore, suggests that the improvement in endothelial function after cocoa

consumption is not solely due to the theobromine content of cocoa. Cocoa also contains other bioactive compounds that may affect FMD, such as epicatechin.[2] Furthermore, it is possible that synergistic effects of the different bioactive components in cocoa have caused the beneficial effects on FMD and RHI. Indeed, Sansone et al. have recently shown that a combination of theobromine (111 mg) and caffeine (11 mg) did not change the FMD, while flavanol consumption (820 mg) alone increased the FMD. When the flavanols were consumed together with the mixture of theobromine and caffeine, circulating concentrations of flavanol metabolites were increased, while the FMD improved even more.[5]

Although theobromine did not change the FMD, we observed an unexpected increase in brachial artery diameters during the postprandial phase. Unfortunately, most studies investigating the effects of cocoa did not report effects on brachial artery diameters. However, one acute study showed an increase in the brachial artery diameter after flavonoid-rich dark chocolate consumption, but with a simultaneous increase in FMD values.[30] We can only speculate about the mechanism underlying the increase in brachial artery diameter. First, theobromine inhibits cyclic adenosinemonophosphate (cAMP)-phosphodiesterase,[31,32] which increases cellular cAMP levels. As a response, intracellular calcium concentrations may decrease, followed by dilatation of the skeletal muscle vasculature.[33] A second potential explanation relates to the increased postprandial insulin responses after theobromine consumption, as we have already earlier reported.[7] Insulin is known to cause vasodilatation of the larger arteries,[34] leading to enlarged artery diameters.

Theobromine did not change fasting and postprandial PWV, but decreased fasting and postprandial AIx. This suggests that effects on parameters reflecting arterial stiffness are divergent, as has also been reported in other studies.[27,35] Differences in PWV are frequently caused by changes in blood pressure.[36] In agreement with the lack of effect on PWV, theobromine did not change fasting and postprandial blood pressure parameters. Neufingerl et al. also observed no effects of 4-weeks of theobromine consumption on fasting blood pressure.[6] Furthermore, cocoa consumption did not affect postprandial blood pressure[30,37] and PWVcf values.[30] In contrast, the consumption of theobromine-enriched flavonoid-rich cocoa drink for 3-weeks increased fasting blood pressure and postprandial PWVcf, while it decreased postprandial blood pressure in hypertensive patients.[38] Possibly, the difference in theobromine dose and drink composition can explain the discrepancy with our findings, since van den Bogaard et al. used a daily theobromine intake of 979 mg, which was consumed in combination with flavonoids provided by the cocoa.[38] Structural characteristics of the vascular wall also determine PWV.[39] However, as both blood pressure and PWV did not change, it can be deduced that these characteristics were also not changed.

Unrelated to theobromine intake, we observed a postprandial decrease in blood pressure, but the anticipated decrease in postprandial PWV was not observed. However, effects of meal consumption on blood pressure as related to PWV are conflicting. One study has reported an increase in blood pressure and PWVcf after meal consumption;[40] another study a decrease in blood pressure, but no change in PWVcf,[41] while no change in blood pressure but an increase in PWVcr has also been reported.[42] For now, it is not clear what causes the discrepancy between the different studies, but it may relate to differences in the amounts of fat in the test meals between the studies.[40-42]

In our study, theobromine decreased fasting cAIx, but did not change fasting pAIx and cAIx75. This is in contrast with the effects of cocoa, since acute and 4-week dark chocolate consumption decreased fasting cAIx75.[16,17] Except for the effects on fasting AIx, the test meal with theobromine decreased cAIx75 and pAIx. This decrease may be related to the postprandial increase in peripheral artery diameters, as a blood vessel with a larger diameter causes a lower reflection wave, leading to a lower AIx. It can therefore be argued that our findings suggest that the main effect of theobromine is dilation of the small and medium-sized peripheral arteries in the postprandial state. If true, then it is unclear why the PWVcr - a measure for peripheral vascular stiffness - did not decrease after theobromine with meal consumption. Finally, theobromine and meal consumption did not affect the arteriolar and venular diameters in the fundus vasculature. Also, acute dark chocolate consumption had no effect on postprandial arteriolar and venular calibers.[12]

In conclusion, theobromine consumption did not improve fasting and postprandial endothelial function, but increased postprandial peripheral arterial diameters and decreased the AIx. These findings do not suggest that theobromine alone contributes to the proposed cardioprotective effects of cocoa.

Supplementary data

Table 1 *Composition of the test drinks (20ml)*

	Theobromine drink	Placebo drink
Theobromine (mg)	500	-
Microcrystalline cellulose (mg)	-	500
Methyl cellulose (mg)	150	150
Sucralose (mg)	10	10
Sodium benzoate (mg)	100	100
Anise 0.1% (mg)	20	20
Water	Till 20 ml	Till 20 ml

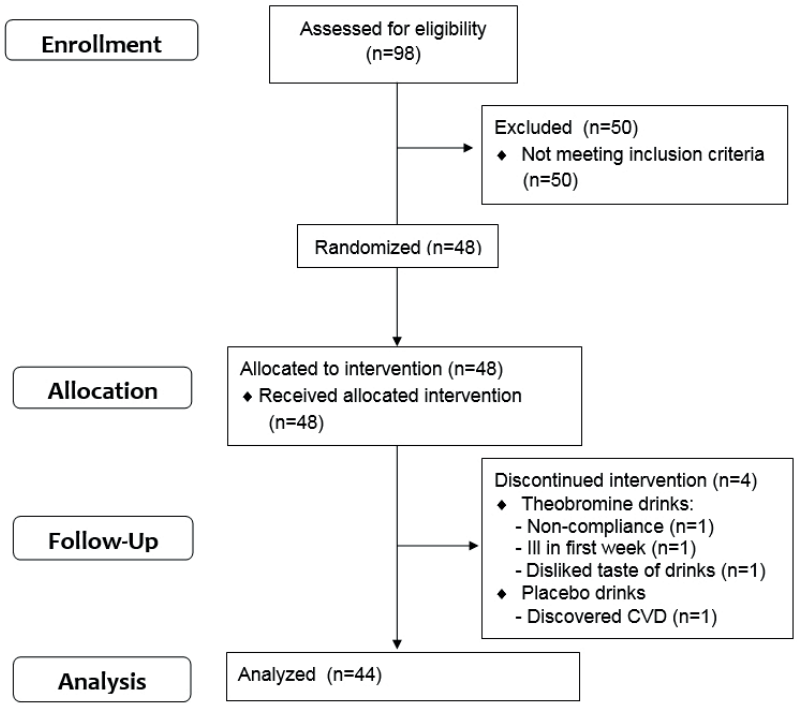


Figure 1 *Flow of participants throughout the study*

Table 2 *Baseline characteristics of the participants that finished the study*[1]

	Mean ± SD
Age (years)	60.3 ± 5.5
BMI (kg/m^2)[1]	29.2 ± 3.0
Total cholesterol (mmol/L)	5.65 ± 0.92
HDL-C (mmol/L)[1]	1.22 ± 0.18
Glucose (mmol/L)	5.56 ± 0.63
SBP (mmHg)[1]	134 ± 15
DBP (mmHg)[1]	86 ± 9
Heart rate (bpm)	70 ± 12

[1] *Values are mean ± SD. n = 44. BMI: Body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure.*

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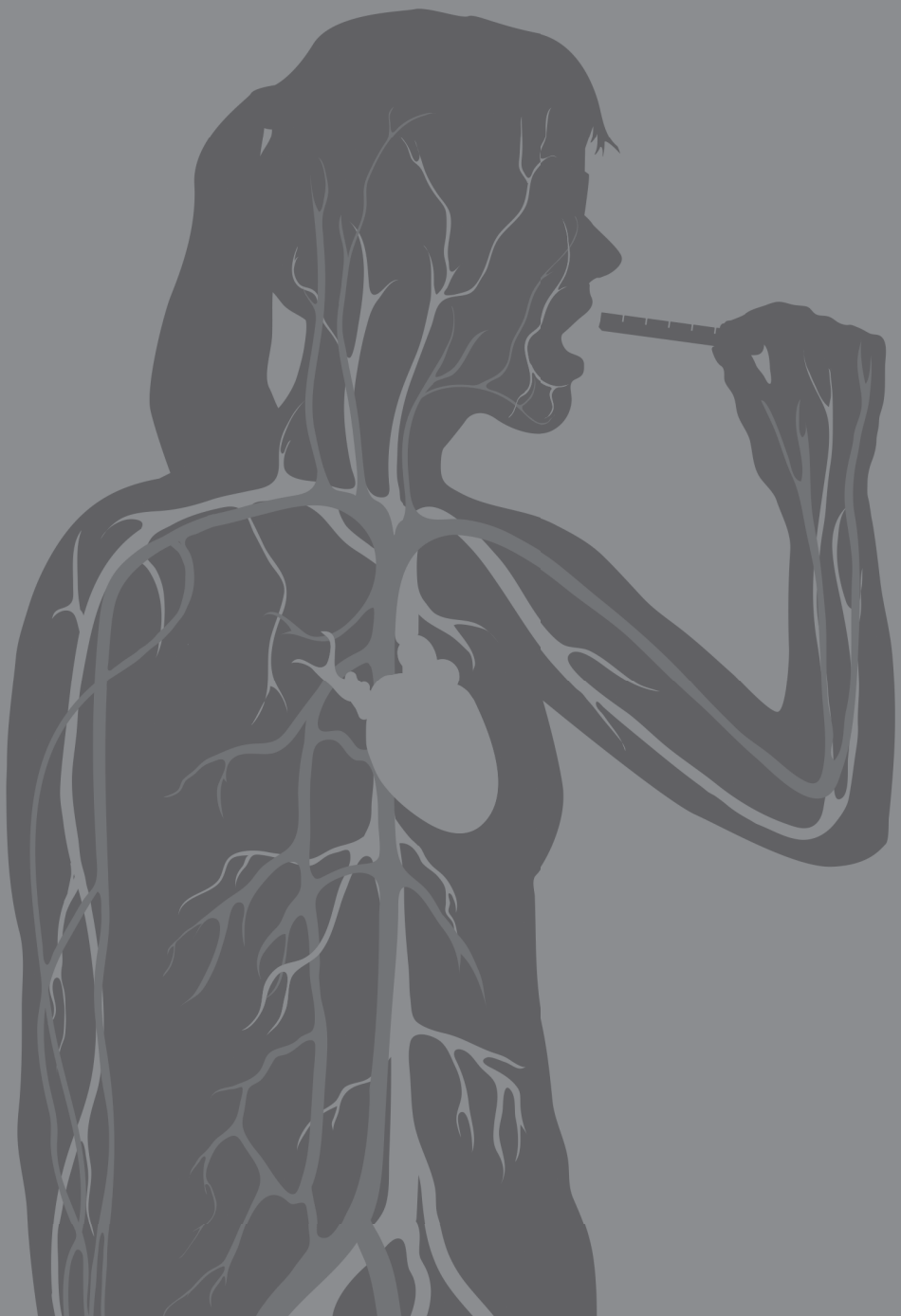
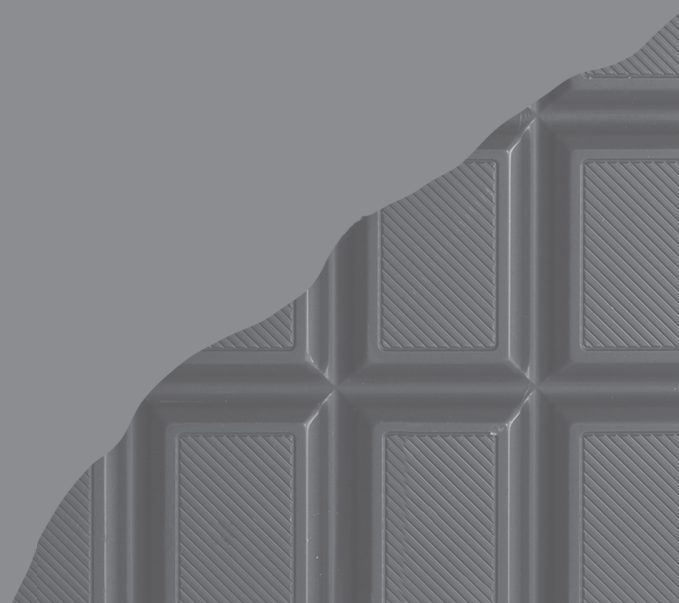


Chapter 7

General discussion



Chocolate and CVD risk

In recent years, the scientific interest for the effects of dark chocolate/cocoa has increased, because of its suggested beneficial effects on human health. High chocolate consumption is associated with lower coronary heart disease (CHD) risk, stroke, cardiovascular events, and cardiovascular mortality.[1] In terms of biomarkers that may explain these potential beneficial effects on disease outcomes, 2-12 weeks cocoa or dark chocolate consumption decreased serum low-density lipoprotein cholesterol (LDL-C) and total cholesterol concentrations,[2,3] increased insulin sensitivity, reduced blood pressure, improved endothelial function and increased serum high-density lipoprotein cholesterol (HDL-C) concentrations.[4]

Increasing serum HDL-C or apolipoprotein A-I (apoA-I) concentrations can be used as a strategy to further lower cardiovascular disease (CVD) risk. The effects of cocoa/dark chocolate consumption on HDL-C concentrations are however contradictory, since two meta-analyses did not show any effects on HDL-C concentrations,[2,3] while one meta-analysis reported an increase in HDL-C concentrations.[4] A stratified analysis, however, found that this increase was only seen in studies with a length of more than 3-weeks,[4] which may explain the different results. Moreover, only one study has investigated the effects of cocoa or dark chocolate consumption on apoA-I concentrations. In that study, it was observed that 4-weeks of cocoa consumption did not change apoA-I concentrations.[5] For now, we can conclude that cocoa may reduce CVD risk, partly because of improved lipid profiles, although effects on HDL-C and apoA-I concentrations are not clear. Since there is currently a rapidly increasing interest in improving HDL functionality to reduce CVD risk over increasing circulating serum HDL-C concentrations, it is unfortunate that no study so far has investigated the effects of cocoa/dark chocolate on HDL functionality.

Given its energy and macronutrient composition, the potential positive effects of chocolate on CVD risk parameters are probably due to one of the minor compounds in cocoa.[6] Since dark chocolate contains more cocoa than other chocolate types, the consumption of dark chocolate should have more favorable health effects than the intake of white or milk chocolate. Indeed, Grassi et al. observed that the consumption of 100 g of dark chocolate for 15 days increased insulin sensitivity, decreased blood pressure, serum total cholesterol and LDL-C concentrations, as compared with white chocolate consumption.[7] Furthermore, Taubert et al. found a decrease in blood pressure, but no change in plasma lipids or glucose concentrations after 18-weeks of dark chocolate compared with white chocolate consumption.[8]

The composition of chocolate

The key ingredients of chocolate are cocoa solids, cocoa butter, sugar, and the emulsifier lecithin. The main commercial chocolate categories are dark, milk and

white chocolate, which differ in the content of the key ingredients. Chocolate contains a high amount of carbohydrates and fat (Table 1), but also various vitamins and minerals (Table 2).

Table 1 *Macronutrient composition of dark chocolate (100g)*[9]

Nutrient	Amount
Protein (g)	6.5
Total carbohydrates (g)	46.7
Mono- and disaccharides (g)	41.7
Polysaccharides (g)	5.0
Total fat (g)	33.7
Saturated fatty acids (g)	20.4
Monounsaturated fatty acids (g)	10.7
Polyunsaturated fatty acids (g)	2.1
Cholesterol (mg)	3.1
Fiber (g)	7.2

Table 2 *Vitamins and minerals in dark chocolate (100g)*

Nutrient	Amount[9]	% of the recommended dietary allowance[10]
Vitamine A1 (μg)	6	0.7
Vitamine B1 (μg)	70	6.4
Vitamine B2 (μg)	90	6.0
Vitamine B3 (mg)	0.8	4.7
Vitamine B6 (μg)	18	12
Vitamine B11 (μg)	15	5.0
Vitamine C (mg)	1	1.3
Sodium (mg)	10	0.6
Potassium (mg)	400	11
Calcium (mg)	50	5.0
Phosphorous (mg)	150	25
Magnesium (mg)	89	25
Iron (mg)	3	33
Copper (mg)	1	111
Selenium (μg)	4	0.07
Zinc (mg)	1	11
Iodine (μg)	3	0.02

Interestingly, several other biologically active components are present in cocoa that may be relevant in the context of CVD risk reduction, including methylxanthines, tocopherols and polyphenols.[11] The content of each macronutrient and micronutrient in chocolate depends on the percentage of cocoa in the chocolate.[12]

Effects of vitamins and minerals in chocolate on CVD risk

An important question is, which component or components in cacao may be responsible for the suggested beneficial cardiometabolic effects. Some of the vitamins and minerals found in dark chocolate may have favorable health effects. Several meta-analyses have investigated the effects of some of these vitamins and minerals in cocoa. It was concluded that a higher intake of sodium increased CVD mortality,[13] that a higher intake of potassium was associated with lower rates of stroke and might also reduce the risk of CHD and total CVD,[14] and that dietary magnesium intake is inversely associated with CVD risk.[15] Furthermore, a randomized trial showed that the B vitamins did not reduce total cardiovascular events.[16] Moreover, studies showed an inverse association between calcium intake and all-cause mortality,[17] and an association between high phosphorus intake with adverse cardiovascular health effects.[18] Because most amounts of the vitamins and minerals in cocoa are small (Table 2), it is not expected that the beneficial effects of cocoa can be ascribed to one of these nutrients. Therefore, the further focus of this discussion will be on other biologically active components of cocoa.

Effects of methylxanthines, tocopherols and polyphenols in chocolate on CVD risk

Xanthines belong to the family of purines and are produced by plants and animals, but also by human cells. Cocoa contains two methylxanthines; theobromine and caffeine (Table 3).[19] Polyphenols are a structural class of compounds characterized by the presence of phenol structures. The flavonoids represent the largest and most diverse group of phenolic compounds found in cocoa. The flavonoids are primarily represented by flavan-3-ols, including (−)-epicatechin and (+)-catechin. Other flavonoids found in cocoa are isoquercitrin, quercetin-3-arabinoside, quercetin and quercetin-3-glucuronide. Furthermore, polymeric condensation products of flavonoids, proanthocyanidins, can also be found in cocoa (Table 3).[11] Tocopherols are natural antioxidants and exist in 4 different isomers; alpha (α), beta (β), gamma (γ) and delta (δ). Generally, the most abundant tocopherols in cocoa are γ-tocopherol, which shows the highest antioxidant activity, and α-tocopherol, the main contributor to vitamin E activity (Table 3).[21]

Table 3 *Methylxanthines, polyphenols and tocopherols in dark chocolate (100g)*[9,19,20]

Compound	Amount
Theobromine (mg)	500-700
Caffeine (mg)	62.5-87.5
Total polyphenols (mg)	1238
(-)-Epicatechin (mg)	115
(+)-Catechin (mg)	26
Isoquercitrin (mg)	6
Quercetin-3-arabinoside (μg)	1750
Quercetin (μg)	550
Quercetin-3-glucuronide (μg)	250
Total proanthocyanidins (mg)	1064
γ-tocopherol (mg)	7.0
α-tocopherol (mg)	0.6

Methylxanthines

Theobromine

So far, only one study has investigated the effects of pure theobromine on serum lipids. The main finding was that theobromine consumption (850 mg) for 4-weeks significantly increased serum HDL-C and apoA-I concentrations (**Chapter 2**), while it decreased those of LDL-C and apolipoprotein B100 (apoB100).[5] The two human intervention studies described in this dissertation investigated the effects of theobromine on fasting and postprandial metabolism and vascular function (Table 4). Furthermore, effects of theobromine on duodenal gene expression were studied to address underlying mechanisms of in particular the increase in apoA-I concentrations after acute and 4-weeks of theobromine consumption (Table 4).

Microarray analysis showed that duodenal gene expression can already be changed within 5 hours after meal consumption, but both acute (**Chapter 4**) and longer-term (**Chapter 5**) theobromine consumption did not change duodenal apoA-I gene expression. Actually, theobromine did not affect the expression of genes and pathways associated with lipid and cholesterol metabolism at all, at least not in the duodenum. Furthermore, acute theobromine consumption lowered duodenal gene expression in relation to glucose metabolism (**Chapter 4**), but this effect was not seen after 4-weeks of theobromine consumption (**Chapter 5**).

Although theobromine did not influence duodenal apoA-I metabolism, there were some clear effects on cardiometabolic parameters. We confirmed the results of Neufingerl et al., that theobromine consumption decreased fasting LDL-C and apoB100 concentrations. However, contrary to their results, fasting HDL-C concentrations only tended to increase, while fasting apoA-I concentrations were not changed at all after theobromine consumption.[5] Furthermore, fully unexpected, we observed a significant increase in fasting high sensitivity C-reactive protein (hsCRP) concentrations after 4-weeks theobromine consumption (**Chapter 5**). Besides these effects, acute and 4-weeks of theobromine consumption did not affect postprandial lipid metabolism (**Chapter 3 and 5**), but increased postprandial insulin (**Chapter 3 and 5**), glucose and free fatty acid (FFA) responses (**Chapter 5**). Our results may therefore suggest that theobromine consumption lowers insulin sensitivity in the postprandial state (**Chapter 3 and 5**). Besides potential changes in metabolic parameters, we also evaluated effects of 4-weeks theobromine intake on parameters reflecting vascular function. In overweight men and women with low HDL-C concentrations, theobromine consumption did not improve fasting and postprandial endothelial function, but decreased the augmentation index (AIx) and increased postprandial arterial diameters. The main effect of theobromine on the vascular system appeared to be dilatation of the small and medium-sized peripheral arteries in the postprandial state (**Chapter 6)** (Table 4). Our results therefore indicate that theobromine can affect fasting LDL-C concentrations, hsCRP, postprandial glucose metabolism and peripheral artery diameters, while it did not change endothelial function and central arterial stiffness.

Since theobromine did not change duodenal gene expression, the effects on lipids, glucose and hsCRP as we observed in our two intervention studies probably originate from another organ, such as the liver. Theobromine is metabolized in the liver by cytochrome P450 2E1 (CYP2E1) and cytochrome P450 1A2 (CYP1A2).[22] Especially the enzyme CYP2E1 may be related to the effects of theobromine, as observed in our studies. Higher theobromine intake might increase CYP2E1 activity,[23] which may cause oxidative stress, leading to lower insulin sensitivity (Figure 1).[24] Furthermore, hsCRP concentrations increased after theobromine consumption. hsCRP is an acute phase protein, which is produced in the liver in response to increased interleukin 6 (IL-6) concentrations.[25] Normally, IL-6 is produced in response to a danger signal. Unfortunately, the studies were not designed to unravel the question why hsCRP increased after theobromine consumption and whether it was caused by increased IL6 reactivity. Elevated hsCRP and IL-6 concentrations can predict the development of type 2 Diabetes Mellitus[26] and are associated with lower insulin sensitivity (Figure 1).[27] Next to these effects, theobromine can inhibit cyclisch adenosinemonophosphate (cAMP)-phosphodiesterase,[28,29] which leads to an increase in cellular cAMP levels

Table 4 *Overview of the main results of the studies presented in this thesis*

Chapter	Type of research	Exposure	Main results and clinical relevance
2	Systematic review	Dietary interventions and novel pharmacological approaches to increase apoA-I concentrations	- ApoA-I can be increased by dietary components, including theobromine - ApoA-I can be increased by novel pharmacological approaches
3	Randomized crossover study with 9 healthy men	Effects of acute theobromine (850 mg), low fat or high fat consumption on postprandial metabolism	- Acute theobromine consumption did not modify the postprandial lipid responses - Acute theobromine consumption increased the postprandial insulin response - High fat consumption lowered postprandial apoB48 responses and increased those of TAG compared with low fat consumption
4	Randomized crossover study with 8 healthy men	Effects of acute theobromine (850 mg), low fat or high fat consumption on duodenal gene expression	- Acute theobromine consumption did not modify duodenal gene expression related to lipid metabolism, but inhibited duodenal gene expression related to glucose metabolism - High fat consumption increased the gene expression related to lipid and cholesterol metabolism and inflammation and lowered those related to glucose compared with low fat consumption
5	Randomized crossover study with 44 overweight or obese subjects with low HDL-C concentrations	Effects of 4-weeks of theobromine (500 mg) consumption on fasting and postprandial metabolism and duodenal gene expression	- Theobromine consumption did not change fasting HDL-C, apoA-I, postprandial lipid concentrations and duodenal gene expression - Theobromine consumption decreased fasting LDL-C, apoB100 and total cholesterol concentrations and increased those of hsCRP - Theobromine consumption unfavorably affects postprandial glucose and insulin responses
6	Randomized crossover study with 44 overweight or obese subjects with low HDL-C concentrations	Effects of 4-weeks of theobromine (500 mg) consumption on fasting and postprandial vascular function	- Theobromine consumption did not affect fasting and postprandial endothelial function - Theobromine consumption increased postprandial arterial diameters and decreased the postprandial augmentation index

ApoA-I: apolipoprotein A-I, apoB100: apolipoprotein B100, apoB48: apolipoprotein B48, HDL-C: high-density lipoprotein cholesterol, hsCRP: high sensitivity C-reactive protein, LDL-C: low-density lipoprotein cholesterol, TAG: triacylglycerol.

in several organs. cAMP plays a role in a number of mechanisms; for example, hepatic glucose production[30] and the exocytosis of insulin granules in the pancreas.[31] This might also have contributed to the observed higher postprandial glucose and insulin concentrations after theobromine consumption (Figure 1). The lower insulin sensitivity can on its turn increase the clearance of HDL-C particles,[32] which translates into lower HDL-C and apoA-I concentrations (Figure 1). Furthermore, elevations of cAMP levels in the muscle cells lead to a decrease in intracellular calcium levels, followed by dilatation of the skeletal muscle vasculature.[33] Additionally, increased insulin concentrations can also cause vasodilatation of the larger arteries.[34] Both these mechanisms may at least partly explain the increase in brachial artery diameter seen after theobromine consumption. Finally, increased arterial diameters can lead to a higher augmentation index (AIx) after theobromine consumption (Figure 1).

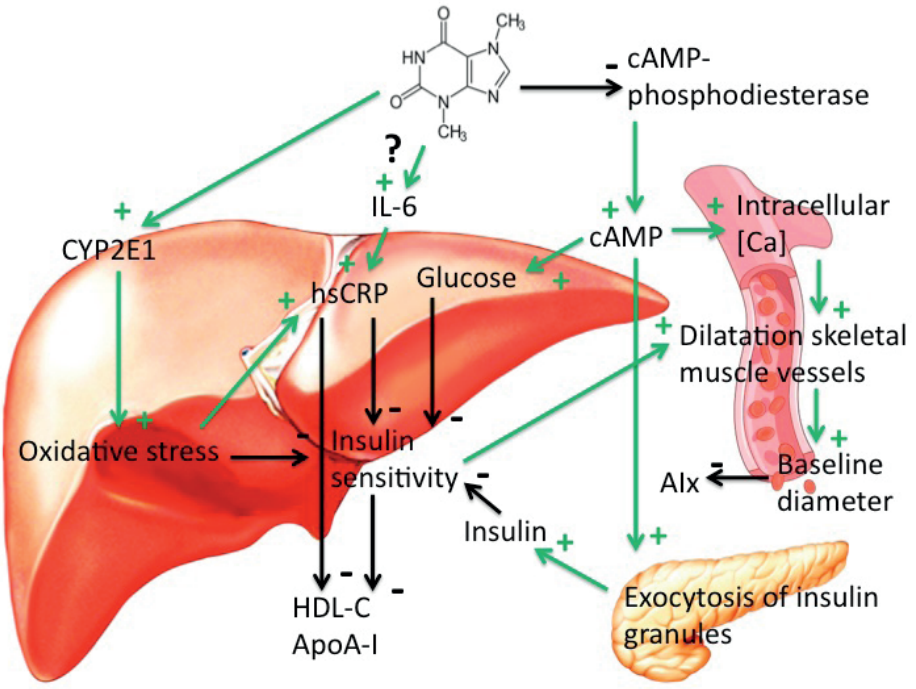


Figure 1 *The main effects of theobromine in the studies described in this dissertation, and the possible underlying mechanisms. AIx: augmentation index, apoA-I: apolipoprotein A-I, [Ca]: calcium concentrations, cAMP: cyclisch adenosinemonophosphate, CYP2E1: cytochrome P450 2E1, HDL-C: high-density lipoprotein cholesterol, hsCRP: high sensitivity C-reactive protein, IL6: interleukin 6.*

The relation between theobromine and HDL-C

Neufingerl et al. have shown a significant increase in both HDL-C and apoA-I concentrations after 4-weeks of 850 mg/d theobromine consumption.[5] To predict the expected increase in serum HDL-C concentrations after the consumption of 500 mg of theobromine a day, in our second long-term intervention study, a dose-response was calculated. Potentially relevant studies published before January 2015 were identified by a systematic search of the database PubMed (www.ncbi.nlm.nih.gov). The following search terms were used to search in titles and abstracts: (Cacao/Chemistry OR Cacao OR Cacao/metabolism) AND (Blood pressure OR blood pressure/drug effects OR Cholesterol, HDL/blood OR Cholesterol, LDL/blood OR Cholesterol, LDL/drug effects OR Cholesterol, LDL/metabolism) AND (humans OR male OR female). The selection of articles was performed in two steps. In the first step, titles and abstracts were screened. Studies were selected if they met the following inclusion criteria: randomized human intervention study with adults, intervention with theobromine/cocoa from which theobromine concentrations could be estimated, and measurement of HDL-C concentrations. In the second step, full-texts of the selected articles were read to extract and estimate theobromine concentrations and to calculate changes in fasting HDL-C concentration. One study was excluded because the intervention and placebo groups were matched for theobromine concentrations.[35] Finally, 12 studies, including 16 data points were included for the dose-response calculations (Table 5). When theobromine intake was not explicitly reported in the article, it was estimated from the cocoa intake. For this the following conversion was used: 100 g cocoa contains 600 mg theobromine.[19] To calculate the dose-response curve, the linear regression procedure was used with the change in HDL-C concentrations as dependent variable and the amount of theobromine as independent variable. This resulted in a regression equation of y=0.0002x (Figure 2).

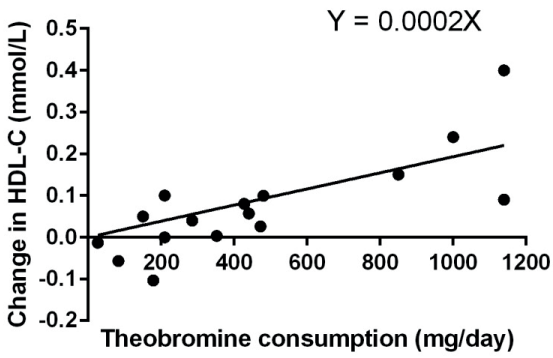


Figure 2 *Dose-response curve of the effect of theobromine on high-density lipoprotein cholesterol (HDL-C) concentrations (12 studies, including 16 data points)*

Table 5 Effect of theobromine consumption on changes in HDL-C concentrations

First author, Year	Intervention	Design, duration	Participants	Theobromine and/ or cocoa intake	Effect on HDL-C
Fraga, 2005[36]	Flavanol containing milk chocolate vs. cocoa butter chocolate	Cross-over, 2 weeks	28 healthy soccer players	178.5 vs. 0 mg theobromine/day	Non-significant 0.10 mmol/L ↓ after flavanol containing milk chocolate compared with cocoa butter chocolate consumption
Grassi, 2005[37]	Dark vs. white chocolate	Cross-over, 15 days	15 healthy subjects	209.5 vs. 0 mg theobromine/day	Non-significant 0.1 mmol/L ↑ after dark compared with white chocolate consumption
Grassi, 2005[38]	Dark vs. white chocolate	Cross-over, 15 days	20 subjects with hypertension	209.5 vs. 0 mg theobromine/day	No change comparing dark with white chocolate consumption
Polagruto, 2006[39]	Cocoa with vs. without phytosterols	Parallel, 6 weeks	67 hypercholesteremic subjects	59.8 vs. 142.6 mg theobromine/day	Non-significant 0.1 mmol/L ↓ after control compared with enriched cocoa consumption
Baba, 2007[40]	Sugar drink with vs. without cocoa powder	Parallel, 12 weeks	25 healthy men	1139.8 vs. 0 mg theobromine/day	Significant 0.4 mmol/L ↑ after cocoa compared with no cocoa consumption
Baba, 2007[41]	0 vs. 13 vs. 19.5 vs. 26g cocoa/ day	Parallel, 4 weeks	160 healthy subjects	0 vs. 285.0 vs. 427.4 vs. 1139.8 mg theobromine/day	Significant 0.04, 0.08, 0.09 mmol/L ↑ after 13, 19.5 and 26 g cocoa compared with no cocoa consumption respectively
Taubert, 2007[8]	Dark vs. white chocolate	Parallel, 18 weeks	44 subjects with hypertension	26.4 vs. 0 mg theobromine/ day	No change comparing dark with white chocolate consumption
Crews, 2007[42]	Chocolate bar and cocoa beverage vs. matched placebo	Parallel, 18 weeks	101 healthy subjects	22 vs. 0 g cocoa /day à 132 vs. 0 mg theobromine/day*	No change comparing cocoa with placebo consumption
Monagas, 2009[43] and Khan, 2012[20]	Skimmed milk with vs. without cocoa	Cross-over, 4 weeks	42 high risk subjects with diabetes mellitus or ≥3 CVD risk factors	440 vs. 0 mg theobromine	Significant 0.06 mmol/L ↑ after cocoa compared with no cocoa consumption
Neufingerl, 2013[5]	Cocoa vs. theobromine vs. cocoa + theobromine vs. control	Parallel, 4 weeks	152 healthy subjects	150 vs. 850 vs. 1000 vs. 0 mg theobromine/day	Significant 0.05, 0.15, 0.24 mmol/L ↑ after 150, 850 and 1000 mg theobromine compared with placebo respectively
Sarria, 2014[44]	Milk with vs. without cocoa	Cross-over, 4 weeks	24 healthy and 20 moderately hyper-cholesteremic subjects	30 vs. 0 g cocoa/day à 180 vs. 0 mg theobromine/day*	Significant 0.05 mmol/L ↑ after cocoa compared with no cocoa consumption in healthy and hypercholesteremic subjects
West, 2014[45]	Chocolate bar and drink with vs. without cocoa	Cross-over, 4 weeks	30 overweight subjects	476 vs. 4 mg theobromine/ day	No change comparing cocoa with no cocoa consumption

Based on this equation, a consumption of 500 mg of theobromine is expected to increase serum HDL-C concentrations with 0.10 mmol/L, but in our study, we found a smaller effect of only 0.04 mmol/L. Furthermore, the only study that investigated pure theobromine found a statistical increase in HDL-C concentrations.[5] Therefore, we expected a significant increase in HDL-C concentrations in our study. There are, however, several suggestions that may explain the discrepancy between the predicted effect on HDL-C concentrations from the dose-response curve, the results of Neufingerl et al., and the actual effect as observed in our study.[5]

The first suggestion is the study population. The dose-response curve included numerous studies that investigated different populations. Several studies focused on totally healthy subjects, while we included healthy participants that were overweight or slightly obese and had relatively low serum HDL-C concentrations. To look more into detail into the study population, a new linear regression line was made with only those studies that did not include healthy subjects.[8,38,39,43-45] This resulted in a regression equation of y=0.0001x (Figure 3), which suggests a lower increase in serum HDL-C concentrations as compared to the total dataset. When we focus on studies which included subjects with a normal BMI[5,8,37,40,41,44] this resulted in an equation of y=0.0002x. These equations suggest that people with a normal BMI are more sensitive to the effects of theobromine on HDL-C concentrations.

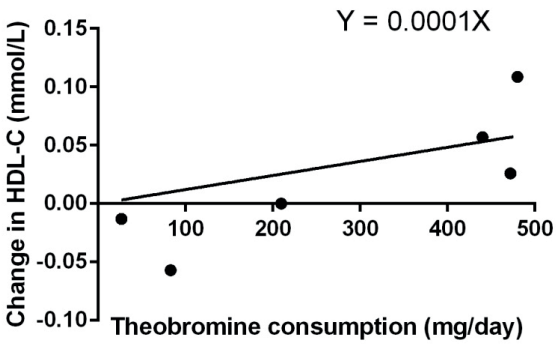


Figure 3 *Dose-response curve of the effect of theobromine on high-density lipoprotein cholesterol (HDL-C) concentrations in a not healthy population (6 studies, including 6 data points)*

With the regression line of the not healthy population, the consumption of 500 mg of theobromine was predicted to increase HDL-C concentrations by 0.05 mmol/L in, which is almost the same as the increase found in our study (0.04 mmol/L). Therefore, it is possible that the study population explains why we did not found a significant increase in HDL-C concentrations, while Neufingerl et al, who included healthy subjects with a normal BMI, did.[5] Maybe the higher body weight in our study

population can explain a part of the differences, since CYP2E1 activity is positively correlated with weight.[46] CYP2E1 is one of the enzymes active in the breakdown of theobromine and caffeine in the liver. Our participants had a higher weight, suggesting that this enzyme is more active, leading to a higher break down of theobromine and lower serum theobromine concentrations. Indeed, when we compare our results with the results of Neufingerl et al., the circulating theobromine concentrations in our study were lower than in their study; i.e. we found serum concentrations around 7 μmol/L with a consumption of 500 mg theobromine, while Neufingerl et al reported concentrations of 30 μmol/L with a consumption of 850 mg theobromine.[5] However, because the theobromine concentrations were measured with different methods, a direct comparison of concentrations must be considered with caution. Comparing the theobromine concentrations at the end of the intervention period of our study between the obese participants (BMI >30 kg/m^2, n=14) and the overweight subjects (BMI < 30 kg/m^2, n=30), showed that the obese subjects had a theobromine concentration of 5.5 μmol/L, while the overweight had a theobromine concentration on 7.9 μmol/L. However, this difference did not reach statistical significance, as tested with an independent t-test. Therefore, the difference in effects between the healthy study population of for example Neufingerl et al, and our participants, is probably not only caused by differences in BMI. However it deserves a more detailed study to prove this hypothesis.

The second suggestion explaining the discrepancy between our results on HDL-C concentrations and the results from the study of Neufingerl et al. can be the difference in time of intake. In the study of Neufingerl et al., the participants consumed the theobromine one hour before breakfast,[5] while in our studies the theobromine was consumed together with a breakfast. The absorption of an oral dose of theobromine is good,[47] but it is unclear if meal consumption affects the absorption, bioavailability and effects of theobromine, therefore this can possibly not explain the differences in effect on HDL-C concentrations.

The third suggestion is the food matrix in which the theobromine is supplied. Neufingerl and colleagues added the theobromine to a milk-based drink,[5] while we dissolved it in water. However, since theobromine is soluble in water, differences in food matrix can probably not explain the differences in effect on HDL-C concentrations. Furthermore, we observed effects on other parameters than HDL-C, and serum theobromine concentrations were significantly increased, although lower than in the study of Neufingerl et al. This means that the absorption and bioavailability of theobromine dissolved in water is good and can induce metabolic effects. It is however possible that theobromine needs one or more components from milk to effectively increase serum apoA-I and HDL-C concentrations. Recently, Sansone et al. showed that a combination of theobromine (111 mg) and caffeine (11 mg) alone did not show

effects on vascular function, while together with flavanols (820 mg) it enhanced the beneficial effects of the flavanols on vascular function, suggesting that the intake of the methylxanthines increased the bioavailability of the flavanols.[48] Although not significantly different, Neufingerl et al. also found higher HDL-C concentrations after theobromine plus cocoa consumption (Δ 0.38 mmol/L) than after theobromine consumption alone (Δ 0.29 mmol/L).[5]

Caffeine

The effects of caffeine on human health have been more extensively investigated than the effects of theobromine. Caffeine is predominately found in coffee, but also in lower amounts in cocoa. A meta-analysis showed that 200-300 mg of caffeine intake leads to an acute increase in blood pressure in participants with hypertension. However, no association was found between habitual caffeine consumption and CVD risk.[49] Furthermore, the replacement of regular coffee by decaffeinated coffee has no effect on serum total cholesterol, HDL-C and TAG concentrations.[50] Additionally, the results of caffeine on flow-mediated dilation (FMD) are contradictory; one study found that acute caffeine ingestion significantly increased the FMD,[51] while two other studies showed that acute caffeinated coffee consumption decreased the FMD.[52,53] Furthermore, **chapter 2** showed that caffeine consumption does not change fasting apoA-I concentrations. In our 4 week intervention, caffeine concentrations increased at the end of the intervention period. However, since caffeine does not show consistent effects on our study outcomes, it is not likely that the effects seen after theobromine consumption are caused by the increase in caffeine concentrations.

Polyphenols

The flavonoids represent the largest and most diverse group of phenolic compounds found in cocoa, with epicatechin and catechin as main forms. A meta-analysis showed that consumption of flavonoid rich cocoa has several beneficial cardiometabolic effects including a decrease in systolic blood pressure (SBP), HOMA-IR and LDL-C concentrations and an increase in FMD and HDL-C concentrations. However, flavonoid-rich cocoa consumption did not change diastolic blood pressure (DBP) and glucose, hsCRP, total cholesterol and TAG concentrations.[54] Additionally, one cohort study showed that a high intake of flavan-3-ols was associated with a 17% lower risk of CVD mortality[55] and another one found a 51% lower risk of CHD mortality.[56] However, three other cohort studies did not found effects of flavanols on CVD or CHD outcomes.[57-59] More studies are therefore needed to investigate which flavanols are associated with lower CVD risk, at which dose and in what population.

Interestingly, the composition of cocoa flavanols appears to influence its effects, particularly on blood pressure. Dark chocolate consumption may be 8 times more

effective in the reduction of the SBP than a cocoa powder drink with a similar dose of flavanols.[6] Comparable results were recently found. Sansone et al. showed that flavanol consumption alone increased FMD and decreased DBP, but when these flavanols were consumed together with a mixture of theobromine and caffeine, the absorption of the flavanols was enhanced, and the FMD increased and the DBP decreased even more.[48] Therefore, it is important to find the optimal effective flavanol composition.

Epicatechin

A meta-analysis showed that epicatechin significantly improved FMD.[4] Epicathechin can upregulate both acute and chronic nitric oxide (NO) production.[60] In contrast to effects on FMD, epicatechin did not change arterial stiffness as measured with pulse wave velocity and AIx.[61] Moreover, a meta-analysis showed that doses of more than 50 mg/d decreased the SBP and DBP, while glucose and TAG concentrations were lowered at moderate doses (50–100 mg/d).[4] Furthermore, epicatechin could improve insulin resistance[62] and may be anti-inflammatory as it lowers Nuclear Factor κ B activation.[63]

Catechin

Catechins from cocoa have been far less studied than the various flavanols and epicatechin. A meta-analysis showed that catechin could significantly increase FMD,[64] and a cohort study showed that catechin intake was inversely associated with ischemic heart disease mortality, but not with the incidence of myocardial infarction and stroke incidence or mortality.[56]

Tocopherols

The most abundant tocopherols in cocoa are γ-tocopherol and α-tocopherol, which are both forms of vitamin E. α-Tocopherol, the major form of vitamin E, received most attention so far, and exhibited several anti-inflammatory, antioxidant and antiatherogenic effects.[65] It may decrease lipid peroxidation,[66] monocyte proatherogenicity, platelet aggregation[66,67] and smooth muscle cell proliferation.[67] Furthermore, α-tocopherol can improve vascular homeostasis via the upregulation of endothelial nitric oxide synthase and NO formation.[68] However, although α-tocopherol exhibits all these beneficial effects in vitro, α-tocopherol supplements have failed to reduce atherosclerosis-related events in human trials,[65] which increased the interest in other members of the vitamin E family. Especially γ-tocopherol exerts all properties of α-tocopherol, but than in a more potent fashion.[65] In a prospective cohort study plasma γ-tocopherol concentrations have been shown to be inversely associated with increased CVD mortality[69] and incidence of death from stroke.[70] However, clinical

trials about the beneficial effects of γ-tocopherol on CVD risk markers are lacking and are needed before conclusions about the effects of γ-tocopherol can be drawn.

Overall conclusion and future directions

Based on the results of the studies presented in this dissertation, it is not likely that the potential beneficial effects of cocoa on metabolic health and CVD can be ascribed to theobromine alone. Acute theobromine consumption does not change postprandial lipid metabolism and duodenal gene expression. Longer-term consumption does not improve fasting HDL-C, apoA-I, or postprandial lipid concentrations, duodenal gene expression and endothelial function, but it unfavorably affected postprandial glucose and insulin responses. This means that other - or combinations of - components from cocoa are more likely candidates to explain the suggested beneficial metabolic effects of cocoa. Besides theobromine, also caffeine and tocopherols in cocoa are probably not the components from cocoa that cause the positive effects on CVD risk, but the flavonoids are likely candidates.

Our studies do not indicate that theobromine alone plays a role in cardiovascular risk management. The question then arises if no further studies with theobromine are warranted at all. In this respect, it is important to realize that bioactive components may exert synergistic effects. As already discussed, a mixture of flavanols with theobromine and caffeine increased the FMD even more than the consumption of flavanols alone.[48] The food matrix may also be of importance. This not only relates to synergistic effects with other components, but also to bioavailability. For flavanols, it has been suggested that dark chocolate consumption more effectively lowers blood pressure than a cocoa powder drink.[6] It is therefore possible that theobromine combined with other compounds or in a different matrix, for example when added to a chocolate bar, has other effects than when in an aqueous solution. Moreover, the population studied may be important, since the effects of interventions can differ between population groups. Although our calculations do not provide definite answer, our regression analysis did suggest that effects of theobromine may differ between population groups (Figures 2 and 3). Another important aspect is the focus of the measurements. Except for the earlier reported favorable effects of theobromine on LDL-C and total cholesterol, we found potentially unfavorable effects on hsCRP after 4-weeks of consumption. In addition, we observed in our acute and long-term study unexpected effects on parameters related to postprandial glucose metabolism. This indicates that both acute and long-term studies are relevant, and postprandial measures should be included in future studies to better identify metabolic effects of interventions. Functional measures, including vascular function measurements and

measures for HDL functionality, are also important to study in future intervention studies related to CVD risk reduction. It is however also important to establish the predictive value of the vascular function measurements in the postprandial state on cardiometabolic health. To better understand and be able to explain effects in more detail, underlying mechanisms should be evaluated. We here focused on the intestine, which is an important organ to understand the absorption and physiologic effects of nutrients. Given its major role in metabolism, the liver is another organ of interest that warrants attention in future studies. However, in contrast to intestinal biopsies, the availability of liver biopsies in relatively healthy populations is limited. This can be partly overcome by using in vitro studies. Hepatocytes can be used as a screening tool to identify natural compounds that can increase apoA-I production. These assays may provide guidance to establish the safety, tolerability and health effects of promising natural compounds in well-controlled human intervention studies.

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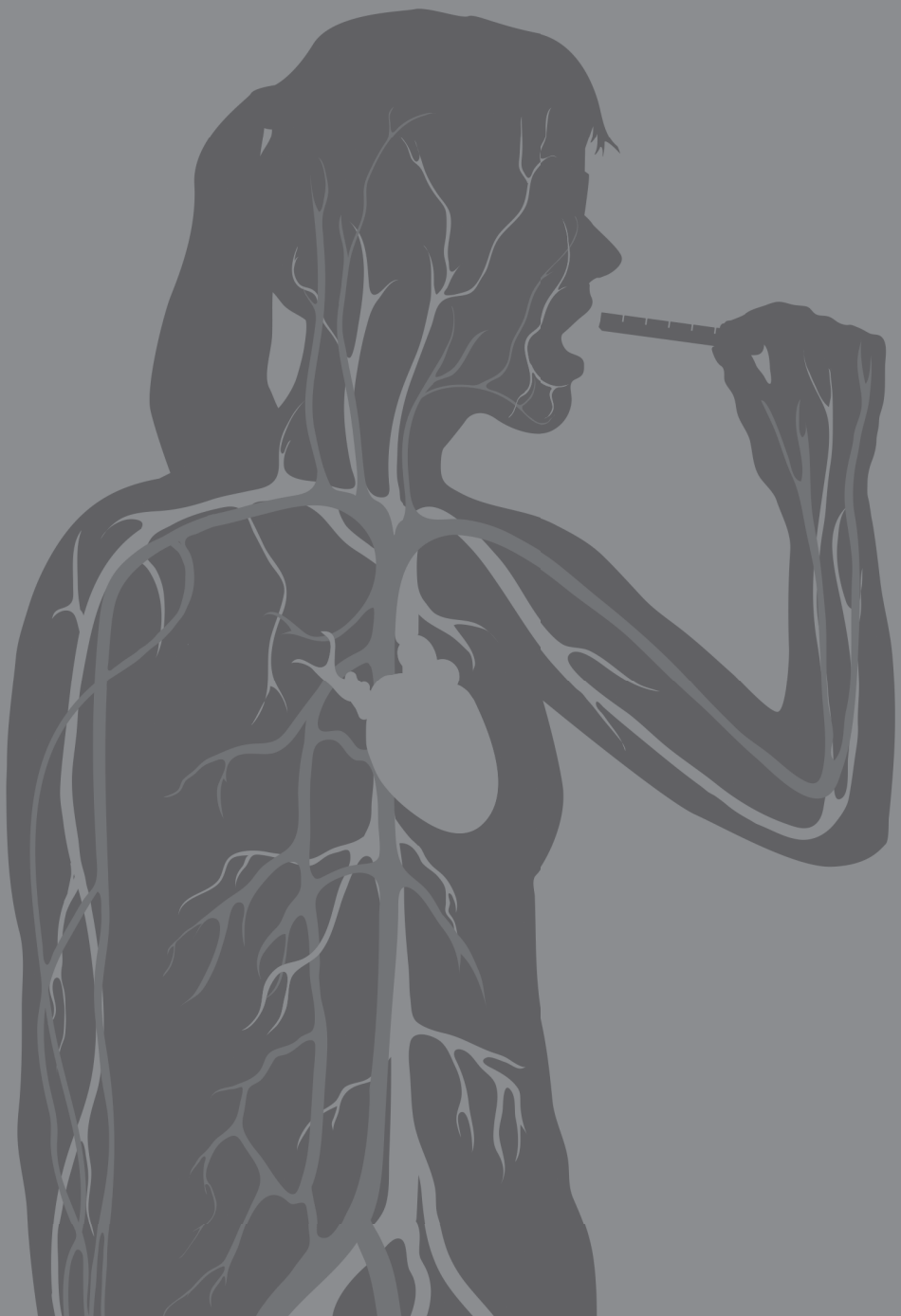
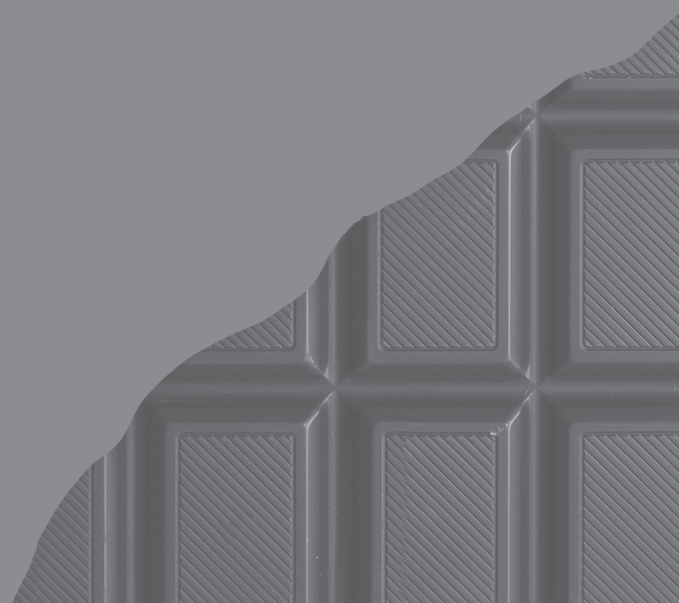


Summary

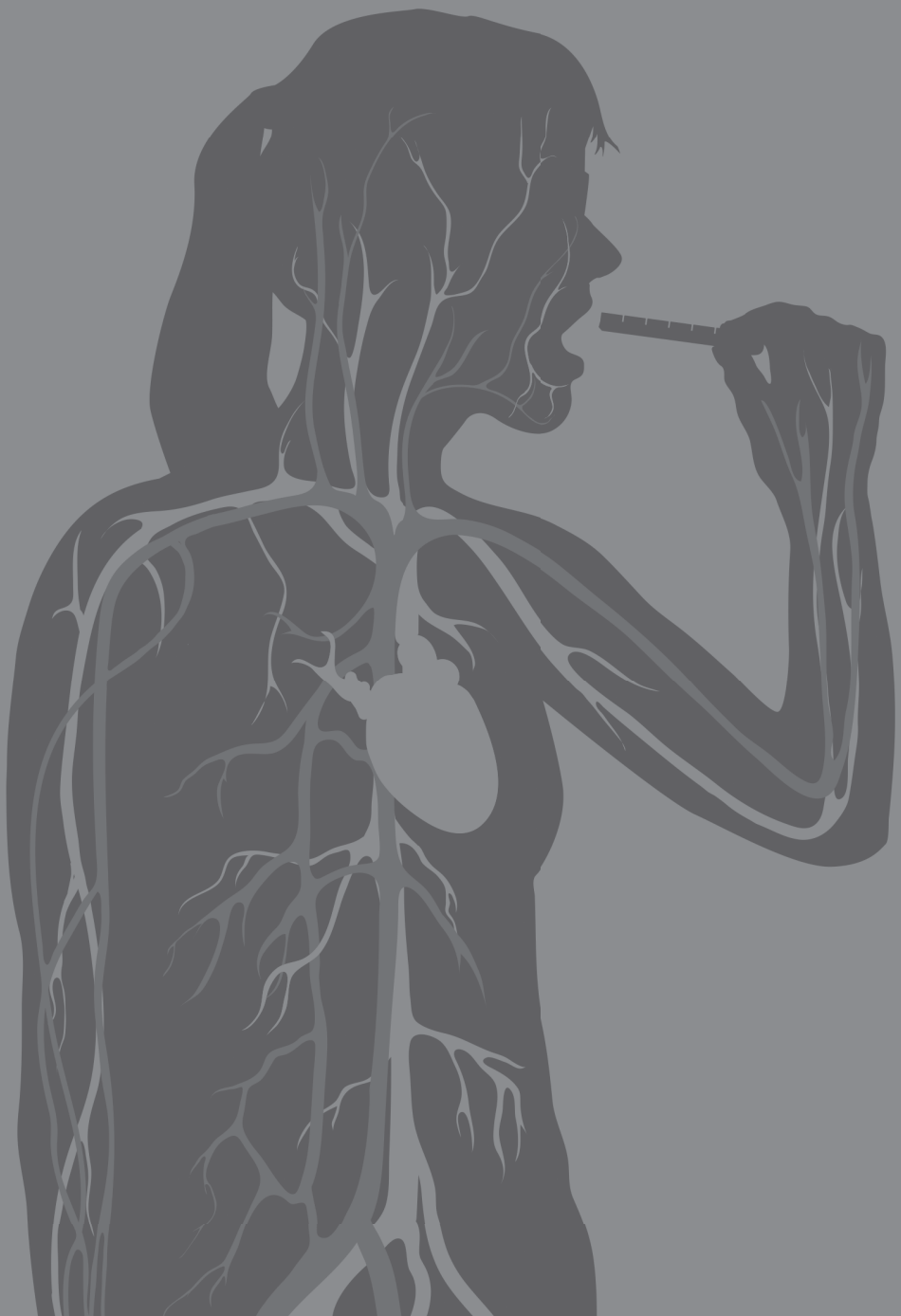


Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the Western world. A healthy diet can be helpful to prevent or delay the development of CVD. Cocoa and dark chocolate consumption have been shown to beneficially affect not only plasma lipids and lipoproteins, but also other CVD risk markers. It is possible that these positive effects are explained by theobromine, which was described to elevate high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (apoA-I) concentrations. Increasing apoA-I concentrations may increase HDL functionality, which can reduce CVD risk. No other studies have investigated the effects of theobromine on other CVD risk markers including postprandial metabolism and vascular function. Additionally, it is interesting to evaluate potential underlying mechanisms for the increase in apoA-I concentrations. The research presented in this thesis therefore focused on (i) a systematic review to identify dietary and pharmacological interventions that increase apoA-I concentrations (ii) two well-defined dietary intervention trials, which assessed the effects of theobromine on CVD markers, including fasting and postprandial metabolism and vascular function. Furthermore, in the intervention studies underlying mechanisms for the increase in apoA-I were investigated by studying gene expression in the duodenum.

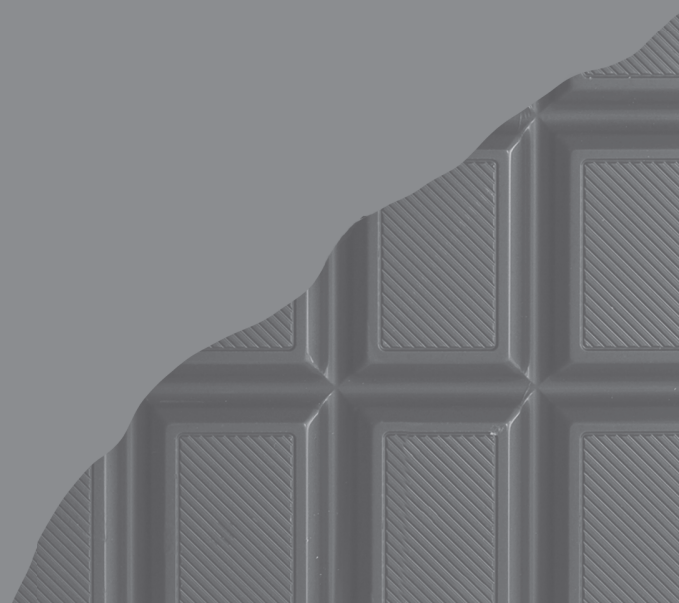
In **Chapter 2**, effects of various nutrients, food components and novel pharmacological approaches targeting apoA-I metabolism were systematically reviewed. Both dietary components and pharmacological approaches can be used to increase apoA-I concentrations. For the dietary components in particular, more knowledge about the underlying mechanisms is necessary, as increasing apoA-I per se does not necessarily translate into a reduced coronary heart disease risk. **Chapters 3** and **4** describe the results of an acute randomized, double blind crossover study. Our hypothesis was that acute consumption of high-fat and of theobromine increased postprandial apoA-I concentrations, when compared with a low-fat meal. We included 10 healthy men who consumed in randomized order a high-fat, low-fat or a low fat meal with added theobromine. After meal intake, blood was sampled frequently for 4-hours. Five hours after meal intake duodenal biopsies were taken for microarray analysis. In **Chapter 3**, we concluded that acute high-fat and theobromine consumption did not change postprandial apoA-I concentrations. Surprisingly, acute high-fat consumption increased triacylglycerol responses but increased postprandial apolipoprotein B48 concentrations less pronounced as compared with low-fat consumption, suggesting the formation of less, but larger chylomicrons after high-fat intake. Interestingly, except for an undesirable increase in the incremental area under the curve for insulin, acute theobromine consumption did not modify the postprandial responses of the low-fat meal. In **Chapter 4**, we concluded that the acute consumption of high-fat and theobromine did not change duodenal apoA-I gene expression. Both theobromine

and high-fat consumption inhibited gene expression related to glucose metabolism. Furthermore, high-fat intake activated the expression of genes related to lipid and cholesterol metabolism and inflammation in the duodenum. **Chapters 5** and **6** describe the results of the second randomized, double-blind, placebo controlled crossover study, in which we hypothesized that 4-weeks theobromine intake improves fasting and postprandial lipid metabolism and vascular function. Forty-four healthy men and women, with low baseline HDL-C concentrations, consumed 500 mg theobromine or placebo daily. After 4-weeks, fasting blood samples were taken and subjects participated in a 4-hour postprandial test, in which blood was sampled frequently for analysis of parameters related to lipid and glucose metabolism. In a subgroup of 10 men, again duodenal biopsies were taken for microarray analysis 5- hours after meal consumption. Furthermore, vascular function was assessed with measures for endothelial function, arterial stiffness and the microcirculation under fasting conditions and 2.5-hours after a mixed meal challenge. Surprisingly, in **Chapter 5** we showed that theobromine lowered fasting serum low-density lipoprotein cholesterol concentrations, but did not change fasting HDL-C, apoA-I, or postprandial lipid concentrations and duodenal gene expression, and unfavorably affected postprandial glucose and insulin responses. **Chapter 6** showed that theobromine consumption did not improve fasting and postprandial endothelial function, but increased postprandial peripheral arterial diameters and decreased the augmentation index, a measure for arterial stiffness. However, more research is needed to determine the predictive value of these vascular function measurements in the postprandial state.

Taken together, the intervention studies described in this dissertation were designed to assess the effect of theobromine on CVD risk markers and explore underlying mechanisms. Unfortunately, we have to conclude that it is unlikely that theobromine alone is the beneficial compound from cocoa. It is however possible that theobromine in combination with other active compounds from cocoa - for example flavanols - in a different matrix - for example a chocolate bar - or in another population groups can have more favorable health effects. This will need further research.



Samenvatting

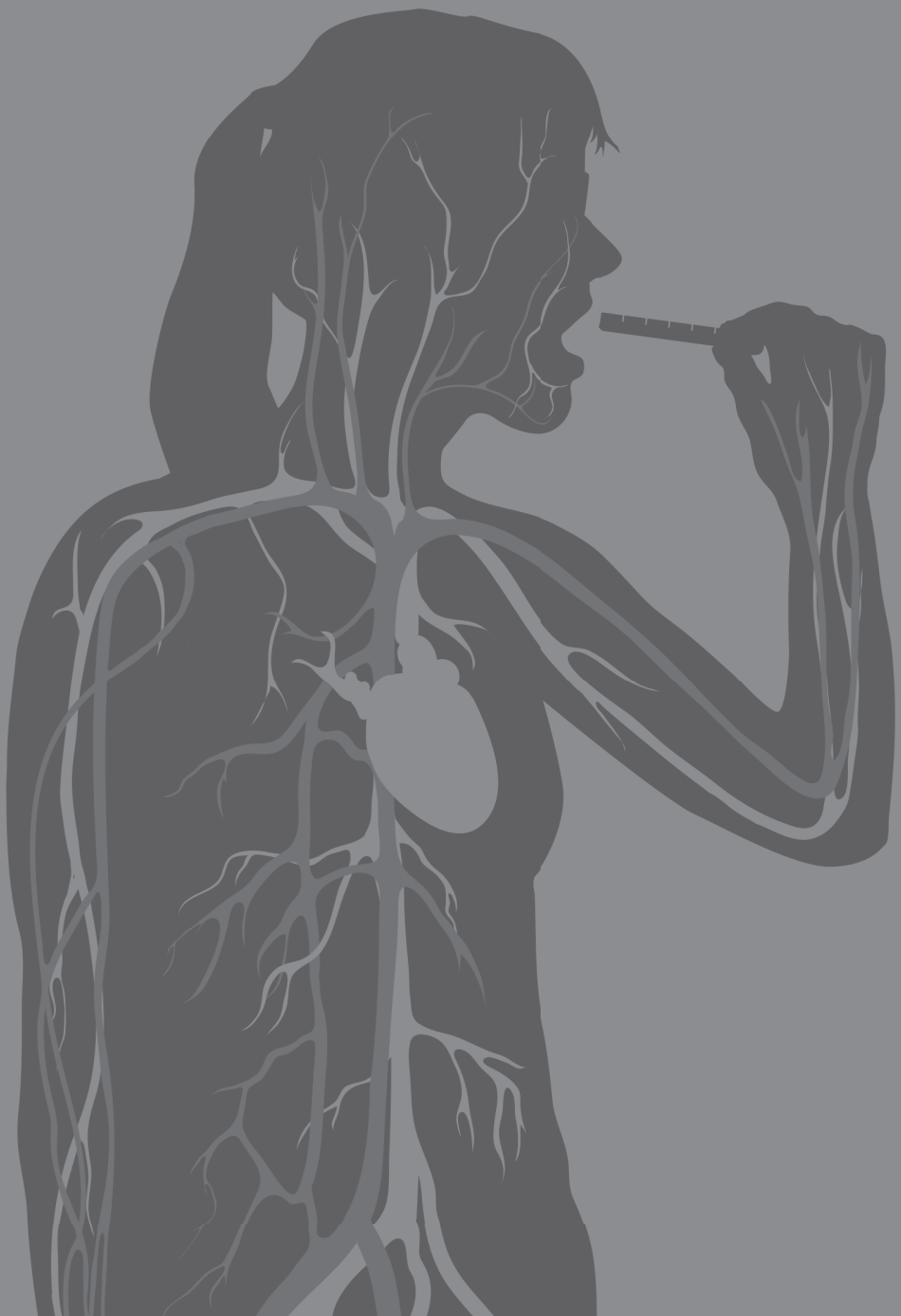


In de Westerse wereld zijn hart- en vaatziekten (HVZ) één van de belangrijkste oorzaken van ziekte en sterfte. Een gezond voedingspatroon is van belang voor de preventie van HVZ. Uit onderzoek is gebleken dat de consumptie van cacao en pure chocolade niet alleen het lipoproteïnenprofiel in het bloed (lipoproteïnen zijn deeltjes die het vet en cholesterol door de bloedbaan vervoeren) verbetert, maar ook andere risicomarkers voor HVZ verbetert. Het is mogelijk dat deze positieve effecten verklaard kunnen worden door theobromine. Dit is een stof, die in cacao voorkomt, waarvoor aanwijzingen zijn gevonden dat het de hoeveelheid apolipoproteine A-I (apoA-I) verhoogt. Het apoA-I is een eiwit, dat in de zogenaamde hogedichtheidslipoproteïnen (HDL) voorkomt. Het verhogen van de apoA-I concentraties kan leiden tot een verbetering van de functionaliteit van een HDL deeltje, hetgeen vervolgens het risico op HVZ kan verlagen. Tot op heden zijn er echter geen studies uitgevoerd, die de effecten van theobromine hebben onderzocht op andere HVZ risicomarkers, zoals de stofwisseling na de maaltijd (het postprandiaal metabolisme) of vaatfunctie. Daarnaast is het belangrijk om onderliggende mechanismen voor het verhogen van de apoA-I concentraties te onderzoeken. Het onderzoek beschreven in dit proefschrift had daarom als doel om door (i) een systematische review voeding en farmacologische interventies te identificeren die apoA-I concentraties kunnen verhogen (ii) het uitvoeren van twee studies om effecten van theobromine op HVZ markers, zowel voor als na een maaltijd (nuchter en postprandiaal), te bestuderen. Daarnaast is onderzoek uitgevoerd naar onderliggende mechanismen om de verhoging van apoA-I concentraties te verklaren. Dit is gedaan door de genexpressie in de darm, een orgaan dat betrokken is bij de productie van apoA-I, te onderzoeken.

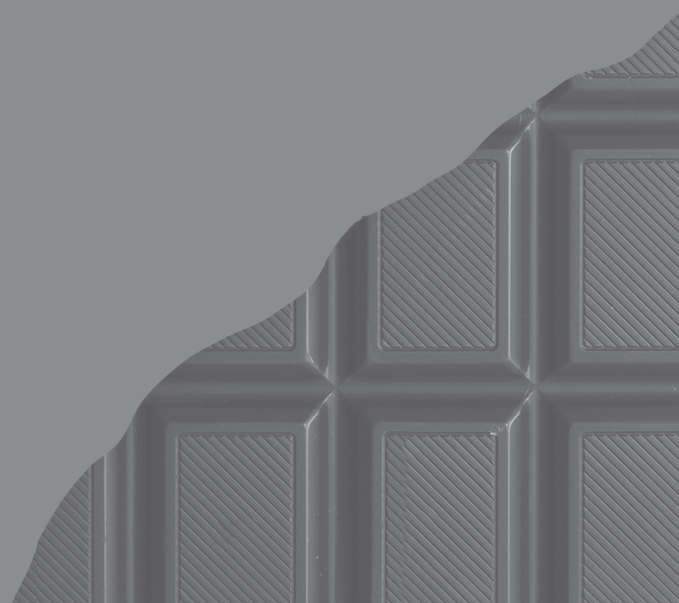
In **Hoofdstuk 2** worden de effecten van verschillende voedingscomponenten en nieuwe farmacologische benaderingen beschreven, die het apoA-I metabolisme kunnen beïnvloeden. We concludeerden, dat voedingscomponenten en farmacologische benaderingen beide gebruikt kunnen worden om apoA-I concentraties te verhogen. Met name voor voedingscomponenten is de kennis omtrent onderliggende mechanismen beperkt. **Hoofdstuk 3** en **4** beschrijven de resultaten van een gerandomiseerde, dubbelblinde cross-over studie. Onze hypothese was dat de éénmalige consumptie van een vetrijke maaltijd of theobromine de postprandiale apoA-I concentratie verhoogt, in vergelijking met een vetarme maaltijd. Aan deze studie hebben 10 gezonde mannen deelgenomen. Zij hebben in willekeurige volgorde een vetrijke, vetarme of een vetarme maaltijd met theobromine, gebruikt. Tot 4 uur na de maaltijd werd regelmatig bloed afgenomen. Vijf uur na de maaltijd zijn er biopten (kleine stukjes weefsel) uit de darm genomen, die gebruikt zijn om de genexpressie in kaart te brengen met behulp van microarray-analyse. In **hoofdstuk 3** concludeerden we dat de vetrijke maaltijd en theobromine geen effecten had op postprandiale apoA-I concentraties in vergelijking met de vetarme maaltijd. Verrassend was dat de

vetrijke maaltijd de hoeveel vetten in het bloed verhoogde, terwijl de postprandiale apolipoproteine B48 concentraties, een eiwit dat in chylomicronen voorkomt, (chylomicronen zijn deeltjes die het vet uit een maaltijd door het lichaam transporteren) verlaagd waren in vergelijking met een vetarme maaltijd. Dit suggereert de vorming van minder, maar wel grotere chylomicronen na een hoge vetinname. Behalve een toename van het insulinegehalte, had het toevoegen van theobromine aan de vetarme maaltijd geen effect. In **hoofdstuk 4** is geconcludeerd dat de éénmalige consumptie van een vetrijke maaltijd en theobromine de apoA-I genexpressie in de darm niet veranderde. Zowel theobromine als de vetrijke maaltijd remden de expressie van genen gerelateerd aan het glucosemetabolisme. Daarnaast activeerde de vetrijke maaltijd de expressie van genen betrokken bij het lipiden- en cholesterolmetabolisme, en ontsteking (inflammatie) in de darm. **Hoofdstuk 5** en **6** beschrijven de resultaten van de tweede gerandomiseerde, dubbelblinde, placebo-gecontroleerde cross-over studie, met de hypothese dat de consumptie van theobromine voor 4-weken het nuchtere en postprandiale vet metabolisme en de vaatfunctie verbetert. Vierenveertig gezonde mannen en vrouwen, met in het begin een lage HDL-C concentratie, gebruikte dagelijks 500 mg theobromine of placebo. Na 4 weken werd er nuchter bloed afgenomen en volgde een deelname aan een 4 uur durende postprandiale test. Tijdens deze test werd regelmatig bloed afgenomen voor analyse van parameters betrokken bij het vet- en glucosemetabolisme. Vijf uur na de maaltijd werden, in 10 mannen, weer biopten uit de darm genomen om de genexpressie in kaart te brengen. Daarnaast werd de vaatfunctie geëvalueerd door het meten van de endotheelfunctie, arteriële stijfheid en de microcirculatie in gevaste toestand (nuchter) en 2,5 uur na een maaltijd (postprandiaal). In **hoofdstuk 5** toonden we aan dat theobromine de nuchtere concentratie cholesterol in de lage dichtheidslipoproteïnen (LDL) verlaagde, maar dat het de HDL-C, apo-AI en postprandiale lipidenconcentraties niet veranderden en geen invloed had op de genexpressie in de darm, terwijl het de postprandiale glucose- en insulineresponsen negatief beïnvloedde. **Hoofdstuk 6** liet zien dat theobromineconsumptie de nuchtere en postprandiale endotheelfunctie niet verbeterde, maar wel de postprandiale perifere arteriële diameters vergrootte en de augmentatie index, een maat voor arteriële stijfheid, verlaagde.

Samengevat, de interventiestudies beschreven in dit proefschrift waren opgezet om de effecten van theobromine op cardiovasculaire risicomarkers te bestuderen en om onderliggende mechanismen te onderzoeken. Uit de studies is gebleken, dat het niet waarschijnlijk is dat het verhogen van alleen de theobromineconsumptie het risico op HVZ verlaagt. Het is echter mogelijk dat theobromine in combinatie met andere stoffen uit cacao - bijvoorbeeld flavanolen - in een andere matrix - bijvoorbeeld een reep chocolade - of in een andere bevolkingsgroep toch gunstige gezondheidseffecten kan hebben. Hiervoor zal verder onderzoek moeten worden verricht.



Valorisation



Social relevance

Non-communicable diseases (NCDs) are chronic diseases, which are not transferred from person to person, but are caused by external factors such as an unhealthy lifestyle. These category of diseases are currently the leading cause of death worldwide, with cardiovascular diseases (CVD) accounting for the highest numbers.[1] Therefore, effective interventions or strategies to prevent or delay CVD development are needed. Since unhealthy diets and insufficient physical activity are key contributors to non-communicable diseases, these two characteristics are key targets in prevention. There are a growing number of foods that target health improvement, the so-called functional foods. The working definition of a functional food is: "a food that is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern."[2] These functional foods can be consumed on a population level and can therefore easily be used in the prevention of CVD. The consumption of functional foods causes small effects on population level, but can have a big impact because many people can consume functional foods. With medicine however, the effects are bigger, but only in a small group of patients, which finally has a lower impact.

Over the years, the scientific interest for chocolate, a food that is extensively consumed in the Western World, has steadily increased. High chocolate consumption is inversely associated with cardiovascular diseases including, coronary heart disease (CHD) risk, stroke, cardiovascular events, and cardiovascular mortality.[3] It is therefore of interest to identify the compound(s) in cocoa that is/are responsible for these beneficial effects. Next, this compound could be isolated from cocoa or potentially other sources and subsequently added to other foods as functional ingredient. Therefore, the studies described in the present dissertation focused on one of the potentially healthy components of cocoa: theobromine. The two intervention studies performed were specifically designed to provide evidence that theobromine can improve cardiovascular health by causing beneficial effects on fasting and postprandial lipids, vascular endothelial function and arterial stiffness and to find the underlying mechanisms for these beneficial effects. Unfortunately, theobromine was found not to be the beneficial component from cocoa. It even had some unexpected negative effects on glucose metabolism and inflammation, and it is therefore not advised to use theobromine as a functional food ingredient for the prevention for CVD risk. It might of course still be possible that in a specific subpopulation theobromine, alone

or in combination with other compounds, has beneficial effects. However, to be used as functional food ingredient it is important that the enriched product can be used without undesirable effects in a wide population.

Economic relevance

Identifying effective interventions to prevent or postpone CVD risk can have an enormous economic impact. CVD represents a major economic burden on health care systems, since it is one of the most costly diseases worldwide. Overall CVD is estimated to cost the Europe economy €210 billion a year.[4] The use of functional foods to prevent CVD can easily be achieved at low cost, which could scale down the medical cost.

Relevance of measurements

The various measurements described in this dissertation may also function as markers to detect the presence of CVD at an early stage. In the Western world, the majority of the population spends a significant part of the day in the postprandial state. Furthermore, increasing evidence suggests that not only fasting lipid, lipoprotein and glucose concentrations, but also a disturbed postprandial lipid or glucose metabolism are important risk markers for CVD.[5] Postprandial measurements are therefore of clinical importance to consider when one studies CVD risk. Also, the vascular function measurements are of clinical importance in the prediction of CVD risk. Flow-mediated dilation is an accepted predictive biomarker for future CVD events.[6] Furthermore, the carotid-femoral pulse wave velocity has already been depicted as a promising future tool for CVD risk prediction in clinical practice.[7] However, more research is needed to determine the predictive value of these vascular function measurements in the postprandial state.

Translation into practice

The finding that theobromine alone is not the compound from cocoa that is beneficial for human health is important for both the industry and science. In science we are a step closer to finding the beneficial compound(s) from cocoa. It is important to realize that with negative findings we also make important progress. Furthermore, for the industry it is clear that theobromine should not be used in functional foods.

The results described in this thesis have been presented at several national and international conferences to colleagues inside and outside the field. We hope to increase the awareness of the medical, societal and economical consequences of CVD and to highlight the potential impact of nutrition in CVD risk reduction. Furthermore, experts of the industrial partners within this project have contributed to the described

research projects through discussions at meetings. Moreover, all research findings have been submitted to international peer-reviewed scientific journals and are therefore accessible to scientists worldwide.

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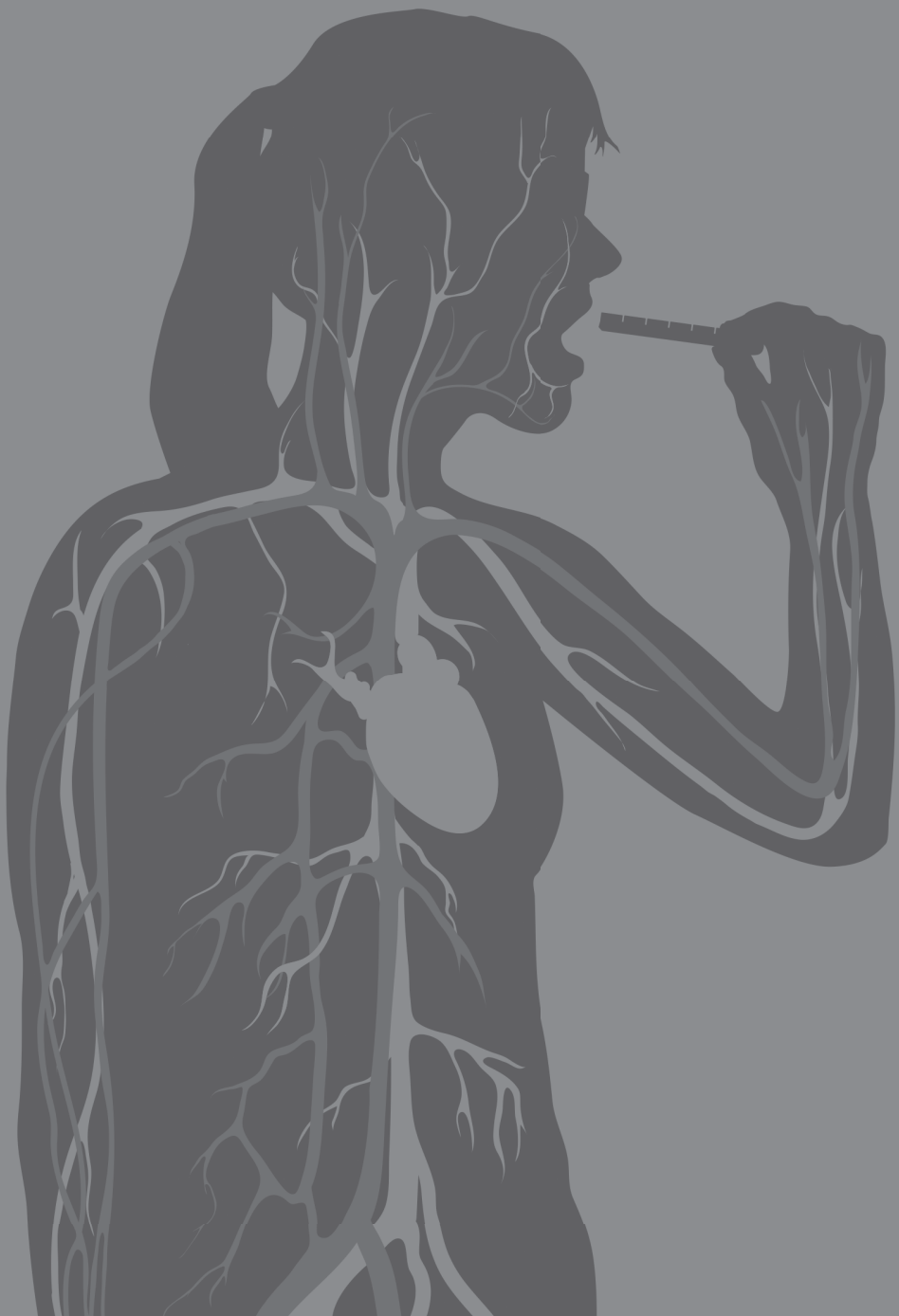
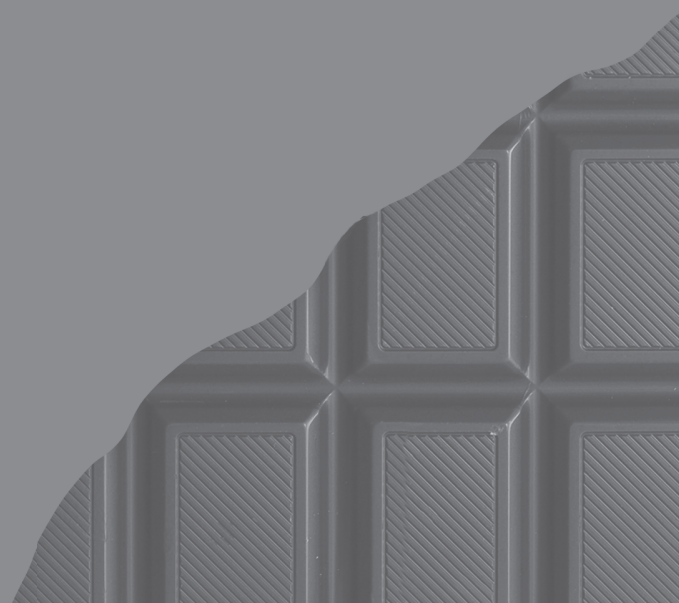


Dankwoord



Zo, wat ben ik blij dat na 4 jaar mijn proefschrift klaar is! Natuurlijk heb ik dit niet alleen gedaan, en ik ben dan ook een heleboel mensen dank verschuldigd. Zonder jullie was mijn boekje nooit zo mooi/goed geworden!

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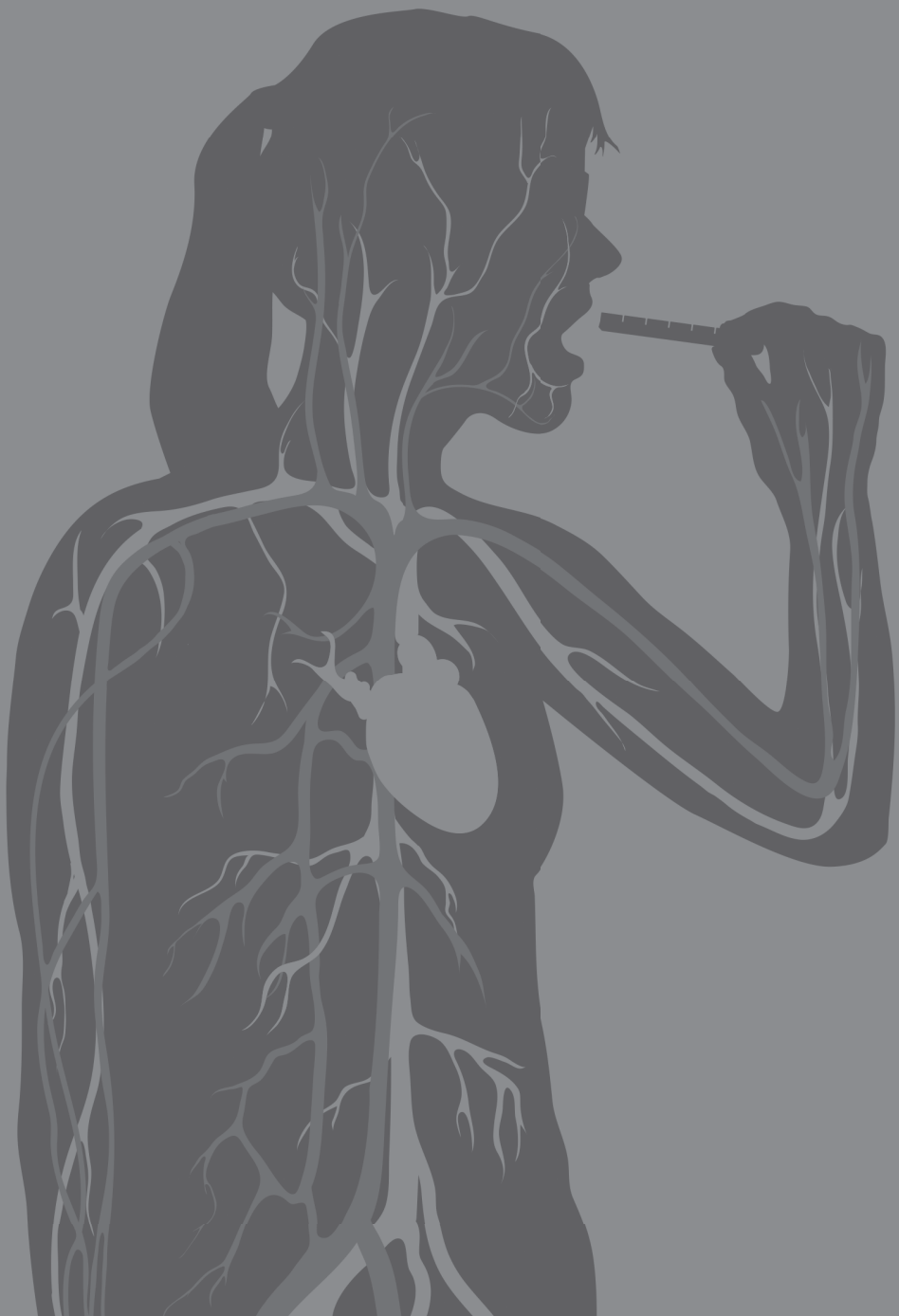
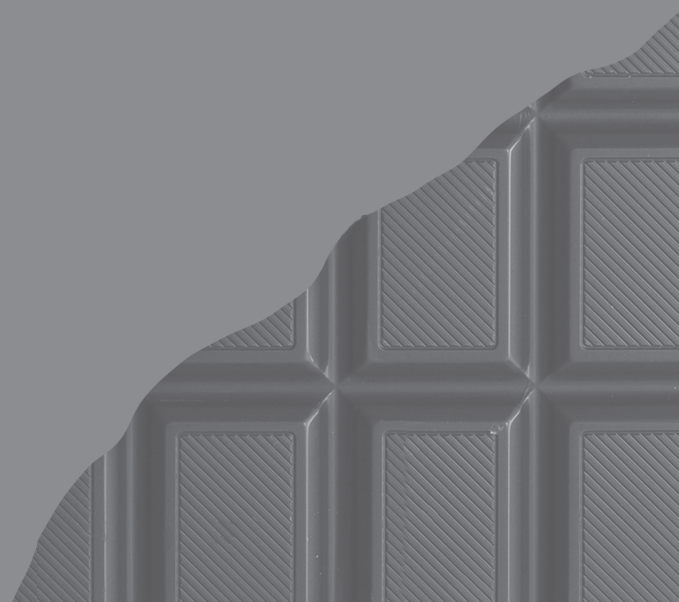
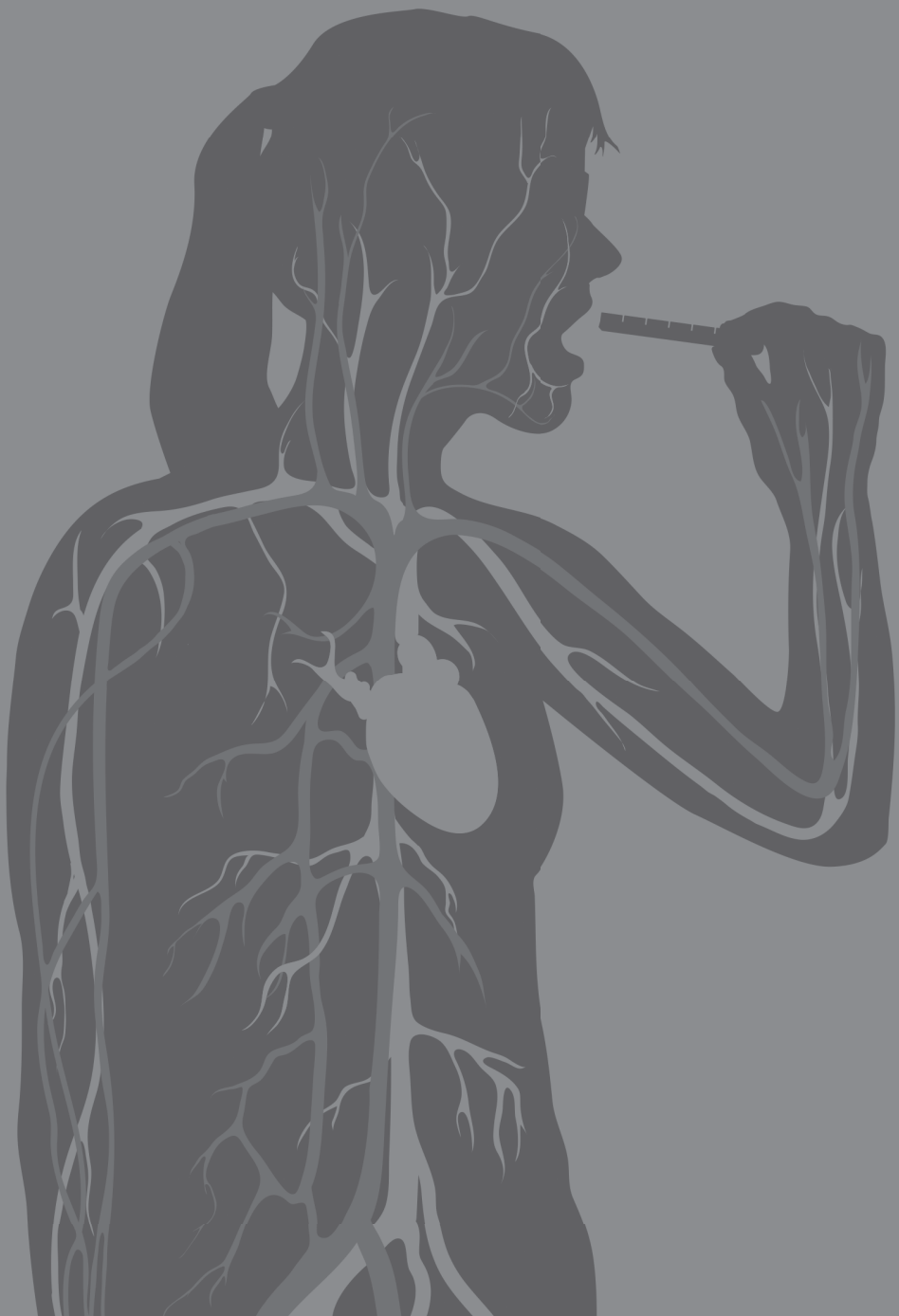


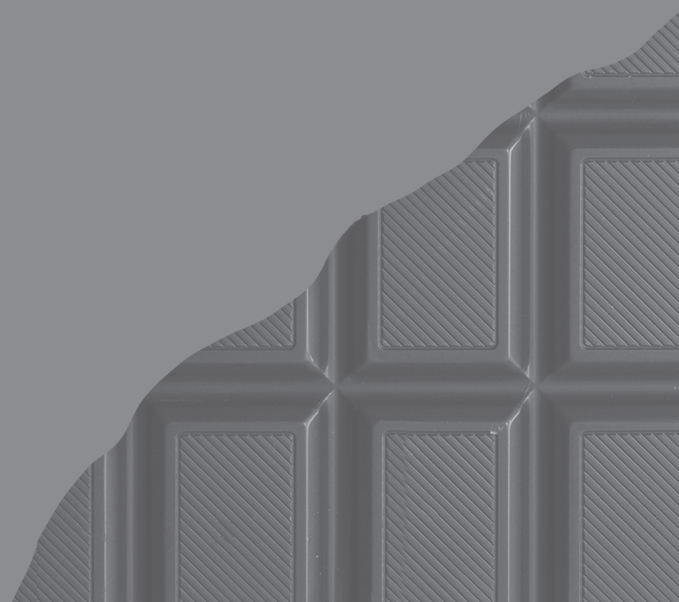
Curriculum Vitae



Lotte Smolders was born on May 18th 1989 in Breda, the Netherlands. She completed secondary school at the Graaf Engelbrecht in Breda in 2007 and she started her study Nutrition and Health at Wageningen University in the same year. She performed two internships during her studies. The first internship was performed at the department of Human Nutrition in Wageningen and the second was a collaboration between the department of Cell Biology and Immunology in Wageningen and the departments of Allergology and Immunology at the Erasmus MC in Rotterdam. She also performed an internship of 6 months at the Nestlé Research Centre in Lausanne, Switzerland. She graduated for the Master of Molecular Nutrition and Toxicology in 2012. In 2013, Lotte was appointed as a PhD-student under the supervision of prof. dr. J. Plat and prof. dr. ir. R.P. Mensink at the department of Human Biology and Movement Sciences at Maastricht University. She conducted several nutritional human intervention trials to investigate the effects of theobromine on human health. During her PhD, she was also involved in teaching and the supervision of students during their thesis. Her PhD project was supported by the Dutch Technology Foundation STW, which is part of the Netherlands Organization for Scientific Research (NWO) and partly funded by the Ministry of Economic Affairs. The research that was performed during this PhD project is described in this dissertation, entitled 'Theobromine: effects on postprandial metabolism, vascular function and intestinal gene expression in humans'. In 2016 she won the 2nd price for the Foppe ten Hoor Young Investigator Award at the Nutritional Science Days (NSD) in Heeze, the Netherlands.



List of publications



Accepted manuscripts

Smolders L, Mensink RP, Plat J. An acute intake of theobromine does not change postprandial lipid metabolism, whereas a high-fat meal lowers chylomicron particle number. Nutrition Research. March 2017

Smolders L, Plat J, Mensink RP. Dietary strategies and novel pharmaceutical approaches targeting serum apoA-I metabolism: a systematic overview

Revision submitted

Smolders L, Mensink RP, Boekschoten MV, de Ridder RJJ, Plat J. Theobromine does not affect postprandial lipid metabolism and duodenal gene expression, but has unfavorable effects on postprandial glucose and insulin responses in humans

To be submitted

Smolders L, Mensink RP, Plat J. Theobromine consumption does not improve fasting and postprandial vascular function in overweight subjects

Smolders L, Mensink RP, Boekschoten MV, de Ridder RJJ, Plat J. The acute effects of dietary theobromine and fat on duodenal gene expression in healthy men

Jacobs DM, **Smolders L**, Yuguang L, Trautwein EA, van Duynhoven J, Mensink RP, Plat J, Mihaleva VV. Effect of theobromine consumption on serum lipoprotein profile in apparently healthy humans with low HDL-Cholesterol